DEVELOPMENT OF BIOGENIC AMINES IN MAHI-MAHI (*Coryphaena hippurus*) AND THEIR CORRELATION WITH SENSORY EVALUATION, TOTAL VOLATILE BASE-NITROGEN, AND PRECURSOR FREE AMINO ACIDS

By

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This is dedicated to my parents Agnes and Clayton, who have taught me the importance of patience, perseverance, and to look for the positive sides of things; my sisters Jane, Brenda, and Albertha, brothers Olgie, Flavian, and Yorick, and wife Joline, for their undaunting support and encouragement of my endeavors.

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Biogenic amines are indicators of seafood quality and spoilage and are formed as a result of decarboxylation of free amino acids in fish, lobster, shrimp, meat, cheese, etc. The decarboxylating enzymes are produced as a result of bacterial growth in the presence of free amino acids. Histamine, one of the biogenic amines, is the causative agent of histamine poisoning which results from eating certain economically important fish species. Evidence shows other biogenic amines potentiate the effects of histamine intoxication. Histamine poisoning is not fatal, but frequently occurs and is a worldwide problem.

Histamine is of importance in mahi-mahi, tuna, and other red muscle fish which typically contain high levels of free histidine. Because free amino acids account, in part, for the rapid spoilage and development of biogenic amines in fish, understanding of

amino acid levels could help explain biogenic amine formation during fish quality deterioration. Therefore, this work was conducted to support development of simplified procedures for judging seafood quality. The approach involved quantification of biogenic amines formed during mahi-mahi storage at refrigeration temperature (7°C) and correlation of the amine levels with their precursor free amino acids and other indicators of fish quality changes. To achieve these objectives, it became necessary to develop a HPLC method which enabled quick analysis of free amino acids in fish. GC was used for analysis of biogenic amines, and steam distillation for volatile base nitrogen (TVB-N). The AOAC fluorometric method was also used for histamine analysis.

The HPLC method used 75% methanol for analyte extraction, o-phthaldialdehyde (OPA) for pre-column derivatization, and solvent gradient elution for analyte determination. Recoveries ranged from 84-91.5 % for lysine, ornithine, histidine and glutamine. The limit of detection for these amino acid standards were in the picomole range.

Results showed significant decreases in the levels of free histidine, lysine, ornithine, and glutamine. Poor correlation ($r = -0.43$, $p < 0.001$) existed between histamine increase and loss of histidine, and between putrescine and ornithine. Histamine levels over time were greater than those of cadaverine. Increase in cadaverine preceded increases in histamine and putrescine, and cadaverine levels were higher than those of putrescine. Good correlation ($r = -0.61$) existed between cadaverine increase and loss of lysine, between cadaverine and histamine ($r = 0.78$), cadaverine and TVB-N ($r = 0.74$), histamine and putrescine ($r = 0.72$), histamine and TVB-N ($r = 0.74$), odor and microbial

level (r = -0.93), and between TVB-N and lysine (r = -0.69). The results showed cadaverine, putrescine and TVB-N are good indicators of chilled mahi-mahi quality and spoilage, and that cadaverine is a good indicator of incipient and late spoilage of mahi-mahi.

CHAPTER 1
INTRODUCTION

The problem of seafood histamine poisoning (also called scombroid poisoning) has engaged the attention of scientists since the mid-1950s (Kimata, 1961; Taylor and Sumner, 1987; Morrow et al., 1991). Still, there is no definitive answer as to all the factors responsible for histamine intoxication (Arnold and Brown, 1978; Chang et al., 1985). It is well known that histamine is the major causative factor of histaminosis (Arnold and Brown, 1978; Lerke et al., 1978; Morrow et al., 1991). However, evidence shows there are so-called potentiators such as cadaverine and putrescine that augment the effect of histamine during scombroid poisoning (Lyons et al., 1983; Taylor et al., 1984; Clifford et al., 1989). As such, scombroid poisoning continues to be a food safety and quality concern to the seafood industry, as well as to scientists and government regulators.

According to a U.S. Department of Health and Human Services report, all foodborne illnesses are under reported to the public health authorities (Gellert et al., 1992; FDA, 1994). From 1973 to 1987, seafood accounted for 4.8% of the reported cases of foodborne illnesses (Appendix A and B). Fifty-five percent of the seafood consumed was imported and not all were thoroughly subjected to customs inspection (FDA, 1994). Sport fishermen catches account for more than 20% of all fish sold in the USA, and

reached restaurants and distributors without being subjected to FDA inspection and control (Gellert et al., 1992). During the period 1979-1989, seafood consumption increased by 23%; however, there was no concomitant increase in reported seafood borne illnesses. Overall, seafood is safe (even safer than other flesh foods in terms of frequency of illnesses, National Academy of Science, 1992), but there is still need for improvement.

Histamine poisoning is generally associated with decomposing scombroid fish such as tuna, mackerel and bonito, and non-scombroid red-muscle fish such as mahi-mahi, sardines, and bluefish (Table1-1) (Taylor et al., 1984; Clifford et al., 1991). These fish are described as red muscle because they contain a high proportion of red muscle tissue. Histamine poisoning is a chemical foodborne intoxication that results from the ingestion of foods containing relatively high levels of histamine (Arnold and Brown, 1978; Lerke et al., 1978; Taylor et al., 1984). It is a world-wide problem that frequently occurs in countries where fish containing high levels of histamine is consumed (Huss, 1994; Smart, 1992; Taylor and Sumner, 1987). Because the disease is generally mild, of short duration and self limiting, it is usually not reported to health authorities. In addition, it is sometimes misdiagnosed as "fish allergy" (Gellert et al. 1992).

Taylor et al. (1984) and Clifford et al. (1991) concluded that histamine formation in marine products tends to be governed by their histidine content. However, there are also incidences of scombroid poisoning involving salmon (Smart, 1992), which may be exceptional among the fish species, because salmon is not a red-muscle fish and not known to have high levels of free histidine. Taylor et al. (1984) and Clifford et al. (1991)

Table 1-1. Fish reported to CDC as vehicles of scombroid poisoning in the United States, 1978-1987[a]

Common Name	Genus	Reported Outbreaks
Mahimahi	*Coryphaena*	55
Tuna	*Thunnus*	41
Bluefish	*Pomatomus*	13
Salmon (raw)	*Oncorhynchus*	2
Marlin	*Makaira*	1
Mackerel	*Scomber*	1
Blue Ulua	*Caranx*	1
Opelu	*Dicapterus*	1
Redfish	*Sebastes*	1

[a] Data not available for 1988-1999.

Source: Seafood Safety, In *Naturally Occurring Poisons*.

also stated that histamine formation is not proportional to the loss of histidine. Fletcher et al. (1995) concluded that histamine-producing bacteria found on fish are capable of producing histidine decarboxylase at ambient temperatures (20-25°C). At that temperature, bacterial activity results in the production of the highest levels of histamine. However, Baranowski et al. (1990) found that both mesophilic and psychrotrophic bacteria were responsible for biogenic amine formation in mahi-mahi and mackerel.

Free amino acids, abundant in protein foods such as fish, also play a major role in the microbial spoilage of fish (Jay and Kontou, 1967; Ingram and Dainty, 1971). They are the immediate precursors of biogenic amines such as histamine, cadaverine and putrescine, all of which are indicators of fish safety and quality (Sikorski et al., 1990). As simple low molecular weight compounds, they provide essential nutrients for the rapid

growth of microbes (Finne, 1992; Jay, 1992). Amino acids are broken down during bacterial spoilage, yielding ammonia, a major TVB-N component, and carbonyl compounds, both of which signal freshness deterioration and spoilage of fish tissue (Schmitt and Siebert, 1961; Liston, 1973).

In order to protect consumers against unwholesome fish, and therefore, possible scombroid poisoning, it is essential to establish controls not only for imported fish but also for the marketed catch of sports fishermen, fish processors, and retailers. Such control mechanisms, e.g Hazard Analysis Critical Control Points (HACCP) plan, as mandated by the government (FDA, 1995b), require stipulation of specific objective parameters of safety, parameters which could influence quality (Kramer, 1966). However, to establish standards and uniformity in measurement and assessment of quality, it is necessary to have objective methods for measuring specific quality attributes. Herein lies the importance of quality indicators such as levels of histamine, cadaverine, putrescine, and total volatile base-nitrogen, compounds which result from amino acid breakdown.

Sensory panels are generally the primary means used to evaluate fish quality; however, they are not always reliable as analytical tools, because of possible subjectivity and variations within the panel (Learson and Ronsivalli, 1969; Hollingworth and Throm, 1982; Bomio, 1998). Consequently, there is always the need for simpler and reliable objective tests which will measure the quality of fish, however quality may be defined (Learson and Ronsivalli, 1969). No practical universal method is available for measuring the overall quality of seafood (Jacober and Rand, 1982). The sensory panel, despite its

limitations, remains the only current method available for measuring the overall quality of fish. However, measures must be taken to control and minimize factors responsible for variability in the sensory data (Kramer, 1966). Jacober and Rand (1982) pointed out that the inherent disadvantages of sensory evaluation range from standardization problems among laboratories to any personal prejudices a judge may have as to color, flavor, and odor.

Given the fact that histamine poisoning is generally associated with the consumption of certain fish species; and because evidence shows that cadaverine and putrescine potentiate the effect of histamine intoxication; we wanted to study the levels of biogenic amines formed in mahi-mahi during storage at refrigeration temperature (7°C). Secondly, we wanted to correlate the levels of the biogenic amines with their precursor free amino acids, sensory quality of fresh and stored fish, and total volatile base-nitrogen (TVB-N). But in order to be able to quantify the free amino acids, it became necessary to develop a HPLC method for amino acid analysis, because the method we were using did not effectively separate the histidine and glutamine peaks. The amino acid analysis was important because they are precursors of biogenic amines, and because of their role in fish spoilage.

Hypothesis. In light of the role of free amino acids in the spoilage of fish; the fact that they are the precursors of free amino acids; and the fact that the absence of histamine is not indicative of freshness or spoilage quality (Mietz and Karmas, 1978); it seems plausible that cadaverine and putrescine, dependent on the presence and levels of the precursor free amino acids, together, would be good indicators of fish freshness or

spoilage, and correlate well with the contents of their precursor free amino acid(s), with TVB-N and sensory evaluation.

CHAPTER 2
LITERATURE REVIEW

Biogenic Amines and Their Formation in Fish Muscle

Fish Muscle

Fish is categorized as white or red muscle fish, based on the level of red muscle tissue present in it (Figure 2-1). Red muscle tissue "is not simply a special tissue with particular properties, but a dynamic material which adjusts its properties" (Love, p 145, 1988). In the majority of fish, red tissue lies immediately under the skin and has a high (5-23%) lipid content (Hultin, 1984). However, in very active fish, such as tuna and mahi-mahi, an additional band of red tissue is located near the spine (Sharp and Pirages, 1978). Chemical differences exist between the white (also called light) and red (also called dark) muscle tissues which are organized into discrete masses (Hochachka et al., 1978). In addition, the variability of the connective tissues, the difference in the size of tissue cells in each myotomic region, and the heterogeneity of the tissues all contribute to the difference between these two tissue types. One major difference between the white and red muscle fish is the high level of free histidine consistently found in the red muscle fish (Lukton and Olcott, 1958). This difference is the basis for red muscle fish being the major fish implicated in histamine (scombroid) poisoning (Takagi et al., 1969).

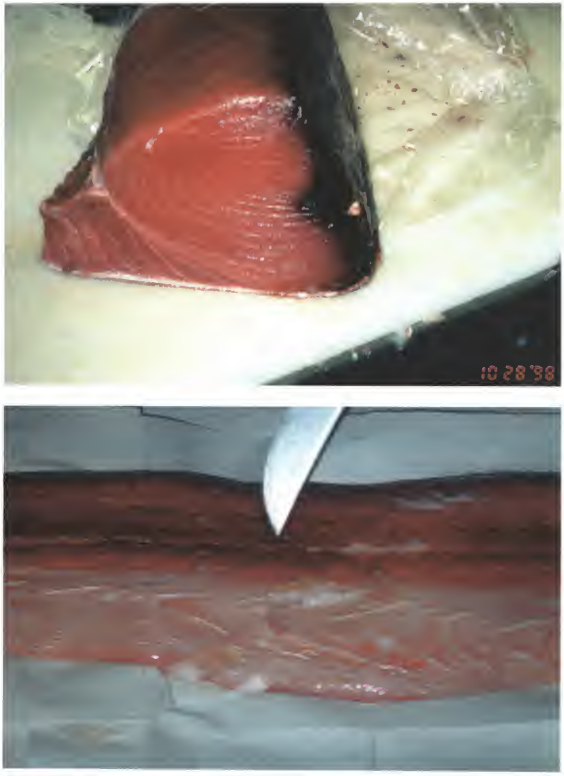


Figure 2-1. Red and white muscle tissues in (A) tuna and (B) mahi-mahi.

Lindsey (1968) and Love (1980) concluded that red muscle tissue in fish is metabolically more active than white because of its greater enzymatic activity. Red muscle tissue contains certain intermediate metabolites, some of which contribute to flavor. For example, they contain heme pigments, mostly myoglobin and hemoglobin, and lipids to supply their energy needs. A larger concentration of mitochondria and DNA per unit weight are found in red muscle tissue because the cells are smaller than white tissue cells. Red muscle tissue also contains a greater concentration of vitamins and most trace elements. More non-protein nitrogenous compounds are found in red muscle tissue which is subsequently richer and more nutritious than white muscle tissue (Mukundan et al., 1979; Shahidi, 1994).

Teleost fish (finfish) have very little carbohydrates (Ashie et al., 1996). Because of this, before and after physical exertion, relatively small changes may occur in the glycogen and lactic acid levels of red muscle tissue, as compared to white tissue. This is probably because the energy needed for contraction of red muscle tissue is derived mostly from lipids, not carbohydrates (Love, 1970). Bilinski (1974) and Hochachka et al. (1978) showed that red muscle tissue is more aerobic than white and can use any form of energy source (carbohydrate or lipid) to operate continuously. Sharp and Pirages (1978) showed evidence that red muscle tissue also utilizes amino acids as a source of energy, something that white tissue seems incapable of doing. They also observed several enzymatic similarities between red muscle tissue and heart muscle which showed that red muscle tissue is adaptable to aerobic metabolic activity. Mukundan et al. (1979) and Love (1970) stated that red muscle tissue seems, in some ways, to resemble the liver tissue of fish, in

terms of its vitamin B level, and resynthesis of energy-rich compounds under aerobic conditions. They too concluded that red muscle tissue has greater enzymatic activity than white muscle tissue.

White muscle tissue, on the other hand, has a greater quantity of certain nucleotides and ATPase. It contains virtually no arginase activity while the superficial red muscle tissue has appreciable activity (Love, 1970). Amino acid distribution is relatively uniform in both white and superficial red tissues (Lukton and Olcott, 1958; Sakaguchi et al., 1982), but a small difference may exist between the deep seated red muscle tissue versus the white. Greater concentrations of glycine, leucine, and phenylalanine are found in the deep seated red muscle tissue, while aspartic and glutamic acids, histidine and lysine are of greater concentrations in white muscle tissue. Our work with tuna and mahi-mahi (Antoine et al., 1999) also showed that white muscle tissue contains greater levels of histidine and lysine than red muscle tissue.

The dipeptides, anserine and carnosine, occur in greater concentrations in the white muscle tissue of active fish than in sluggish species. Taurine is richer in red muscle tissue than white, and is more likely an energy store than an osmoregulator because of its seasonal variations. A greater proportion of carnosine, relative to anserine, is found in fresh water fish versus marine fish. Carnosine is apparently not synthesized by fish; therefore, its source must be the diet, which causes the levels to vary. Similarly, trimethylamine (TMA) is richer in red muscle tissue than in white (Love, 1980), and is present in marine species but not in fresh water species. Love (1970) and Mukundan et al. (1979) concluded that there are numerous chemical differences between red and white

muscle tissues; hence, for comparisons to be useful (Love, 1970), it is important that sampling procedures specify and use the same region of fish tissue.

Histamine Poisoning

Histamine, one of the biogenic amines, is primarily the result of bacterial enzymatic activity on dead aquatic products, meats, and other food products (Beljaars et al., 1998). Histamine production is more commonly the result of high temperature spoilage than long term, relatively low-temperature spoilage (Anonymous, 1997). Histamine, like some other biogenic amines, may have adverse physiological effects in humans. In the case of fish, interest is in histamine (Eitenmiller and De Souza, 1984), cadaverine, and putrescine because of their involvement in scombroid poisoning. Zapsallis and Beck (1986) stated that biogenic amines in foods are potentially dangerous organic molecules which make them a public health concern. Some of these are vasoactive compounds which cause dilation of blood vessels, headaches, nausea, cramps, and a burning sensation in the mouth (Sikorski et al., 1990; Gill, 1990; Taylor, 1988; Zapsallis and Beck, 1986; Arnold and Brown, 1978). These amines transit the gastrointestinal tract unaltered. Their biochemical effects depend on the dietary dosage and/or consumption frequency, and on the potentiators of histamine-like poisoning (Taylor, 1988; Arnold and Brown, 1978).

Symptoms of histamine intoxication are nausea, vomiting, diarrhea, hypotension, headache, tingling of the tongue, burning sensation in the mouth and hives (Taylor et al., 1984; Lerke et al., 1978). These symptoms may appear within several minutes to several

hours after ingestion of the contaminated food. The illness is benign, self limiting, and lasts a few hours, but the symptoms may continue for several days (Gellert et al., 1992; Taylor et al., 1984).

Miyaki and Hayashi (1954a) reported that "a factor" cooperates with histamine to cause scombroid poisoning in dried mackerel pike. They later observed a synergistic effect when trimethylamine-N-oxide (TMAO), TMA, choline, and agmatine, in limited concentrations, were each added to the threshold levels of histamine (Miyaki and Hayashi, 1954b). Mongar (1957) and Lyons et al. (1983) later reported that diamines, produced during fish degradation, potentiate the effects of histamine poisoning because of their competitive inhibition on the enzymic destruction of histamine in the gut. Arnold and Brown (1978) and Taylor (1988), in their reviews, concluded that histamine is not the sole factor responsible for histamine poisoning.

While it is clear that histaminosis is associated with the consumption of fish (Table 2-1) containing high levels of histamine (Lerke et al., 1978; Motil and Scrimshaw, 1970), it is also evident that ingestion of large doses of histamine do not cause histaminosis (Ijomah et al., 1991; Clifford et al., 1989). On the other hand, Ijomah et al. (1991) claim that it is not dietary but endogenous histamine and probably other biologically active substances that are responsible for scombroid toxicity. Meanwhile, evidence indicates other amines are also important in potentiating the onset of histaminosis (Taylor and Sumner, 1987; Foo, 1976; Mongar, 1957; Kawabata et al.,

Table 2-1. Fish species known to commonly cause histamine poisoning.

FAMILY	SPECIES
Scombroidae	tuna, mackerel, cero, and sierra
Pomatomidae	bluefish
Coryphenaenidae	mahi-mahi
Caragidae	jack mackerel, amberjack, yellowtail
Clupeidae	herring, sardines
Engraulidae	anchovies

Source: Taylor and Sumner, 1987, In Seafood Quality Determination by Kramer and Liston.

1955). Cadaverine and putrescine (Luten et al., 1992; Kim and Bjeldanes, 1979), as well as other imidazole containing compounds (Crush, 1970), are examples of such potentiators (Taylor and Sumner, 1987).

It is thought that polyamines are potentiators of histamine-like poisoning because symptoms of scombroid poisoning cannot be reproduced by simply taking histamine orally in the presence of polyamines. The polyamines seem to inhibit diamine oxidase and histidine-N-methyl transferase, histamine detoxifying enzymes in the gut, resulting in increased absorption of histamine (Taylor and Sumner, 1987; Arnold and Brown, 1978).

Scombroid poisoning is preventable, for the most part, because it is caused by histamine which results from bacterial decomposition of fish (Gellert et al., 1992). However, once fish is contaminated with histamine, it is unsafe because cooking does not inactivate the toxin (Frank and Yoshinaga, 1984). As such, Food and Drug Administration (FDA, 1995a) stipulates that 5 mg per 100 mg of histamine in tuna and

mahi-mahi is a defective action level, and 50 mg per 100 mg as the action level (or hazard action level) in all other species known to be implicated in histamine poisoning. The European Community proposes that an average of 10 mg of histamine per 100 g per nine samples, be considered as the defective action level, and no sample may have greater than 20 mg of histamine per 100 g of fish (Luten et al., 1992). Contaminated fish represents food and economic wastes which can be avoided, if proper handling practices are carried out, beginning at the site of the catch to the point of consumption (Hughes and Potter, 1991).

Quality changes in fresh fish occur rapidly and are chiefly a result of bacterial and autolytic activities (Slavin, 1963). Though much work has been done on assessing the quality changes occurring in fresh fish, the application of quality specifications and standards demand the need for better objective criteria and methods to measure various degrees of freshness quality (Osterhaug et al., 1963). Given the unpredictable levels of histamine formation and distribution in fish, the FDA regulations, and industry concern for consumer safety, there is a continuous need to search for simpler and easier methods which can quantify the indicators of fish safety and spoilage qualities.

Histamine Distribution in Fish

Lerke et al. (1978) showed that the distribution of histamine in spoiling tuna is quite uneven, varying more than three to four times over short distances (3 cm) in contiguous areas. Noticeably, the highest concentrations are found near the gut cavity. Thus, the ventral sections yield the highest levels of histamine. This may be expected

since the gut cavity carries a high microbial load, and fish are usually gutted before chilling. Baranowski et al. (1990), on the other hand, found that the pattern of biogenic amine formation is similar at different temperatures, although the levels and rates of formation are slower at lower temperatures. The histamine levels may even form a gradient in which the anterior loin sections have the highest levels, with decreasing amounts approaching the tail. There is no set pattern for the development of histamine in the various regions of the fish. Hence, Etkind et al. (1987) concluded that the concentration of histamine may vary widely from one part of the fish to another; therefore, multiple areas should be sampled, or samples should be homogenized prior to analysis.

Takagi et al. (1971) found that, unlike red muscle fish, histamine in molluscs and crustaceans was either absent or present in small amounts (< 3 mg/100g), even though they considered small as < 10 mg/100g. The rate of decrease of histidine and formation of histamine varied with species. More histamine was formed in the white tissue than in the red tissue of the red muscle fish. They concluded that the degree of histamine formation was related to histidine content but was not directly proportional to the loss of histidine. Fletcher et al. (1995) made the same conclusion.

Takagi et al. (1971), in their study of putrefaction of squid and octopus muscle, observed that in squid, cadaverine and extremely small amounts of putrescine were formed but no histamine or tyramine. Ohashi et al. (1991) found that the level of ornithine in squid muscle increased before levels of agmatine, and in a pattern similar to that of agmatine; whereas in octopus, cadaverine, putrescine, and very small amounts of

tyramine formed during spoilage but no histamine was found (Takagi et al., 1971). While histamine-producing bacteria are few in number (Table 2-2), the producers of putrescine and cadaverine are more widespread (Table 2-3), especially among the Enterobacteriaceae (Taylor and Sumner, 1987; Niven et al., 1981), since many bacterial species produce ornithine and/or lysine decarboxylase. This explains, in part, why cadaverine and putrescine tend to always be present in spoiled fish (Rodriguez-Jerez et al., 1994; Middlebrooks et al., 1988) even when histamine is not always present. This makes it necessary to identify possible correlations of histamine development with other amines and/or indicators, which can be used for fish safety and quality assessment.

Biogenic Amines

Biogenic amines are chemically defined as aliphatic, alicyclic and heterocyclic organic bases of low molecular weight (Luten et al., 1992) (Figure 2-2). These amines may be used to measure the freshness of aquatic food products (Koutsoumanis et al., 1999; Botta, 1994; Yamanaka, 1990; Mietz and Karmas, 1978). Mietz and Karmas (1978) reported that polyamines may be more useful as spoilage indicators than histamine, because of variations in histamine levels even within the same species. These biogenic amines form part of the non-protein nitrogen (NPN) extractives, which are part of the main flavor compounds of fish and other food products (Sikorski and Pan, 1994; Love, 1980; Thomson et al., 1980). Unlike the lipids which form the main part of the flavor of fatty fish (Love, 1980), biogenic amines are water-soluble, and their contribution to the flavor and odor of fish depends on product freshness, the amine

Table 2-2. Histamine-producing bacteria isolated from marine fish.

Organism	Source	Reference(s)
Citrobacter freundii	Skipjack tuna	Taylor and Speckhard, 1983
Clostridium perfringens	Skipjack tuna	Yoshinaga and Frank, 1982
Edwardsiella sp.	Mahi-mahi	Niven, Jeffery and Corlett, 1981
Enterobacter aerogenes	Skipjack tuna	Yoshinaga and Frank, 1982
	Tuna, mahi-mahi	Niven, Jeffery and Corlett, 1981
Enterobacter cloacae	Tuna	Niven, Jeffery and Corlett, 1981
Escherichia coli	Tuna	Ferencik, 1970
	Tuna	Niven, Jeffery and Corlett, 1981
Hafnia alvei	Tuna	Havelka, 1967
	Tuna	Ferencik, 1970
	Skipjack tuna	Omura, Price and Olcott, 1978
	Jack mackerel	
Klebsiella pneumoniae	Tuna	Lerke et al., 1978
	Mahi-mahi, mackerel	Niven, Jeffery and Corlett, 1981
	Tuna	Taylor et al., 1979
	Skipjack tuna	Yoshinaga and Frank, 1982
Klebsiella sp.	Skipjack tuna	Omura, Price and Olcott, 1978
	Jack mackerel	
Proteus mirabilis	Bigeye tuna	Kawabata et al., 1956a
	Skipjack tuna	Yoshinaga and Frank, 1982
Proteus morganii	Bigeye tuna	Kawabata et al., 1956, 1956a
	Fish	Kimata, 1961
	Skipjack tuna	Omura, Price and Olcott, 1978
	Jack mackerel	
	Tuna, mahi-mahi, mackerel	Niven, Jeffery and Corlett, 1981
	Skipjack tuna	Taylor and Speckhard, 1983
Proteus sp.	Tuna	Ferencik, 1970
	Skipjack tuna	Omura, Price and Olcott, 1978
	Jack mackerel	
	Mackerel	Niven, Jeffery and Corlett, 1981

Table 2-2 cont. Histamine-producing bacteria isolated from marine fish.

Proteus vulgaris	Bigeye tuna	Kawabata et al., 1956a
Vibrio alginolyticus	Skipjack tuna	Yoshinaga and Frank, 1982
Vibrio sp.	Mackerel	Niven, Jeffery and Corlett, 1981

Source: Frank, H.A., 1985, FAO Fish. Technol. Paper 252.

Table 2-3. Bacterial species possessing lysine or ornithine decarboxylase.

Lysine decarboxylase	Ornithine decarboxylase
Edwardsiella hoshinae	*Cedecea davisae*
Edwardsiella ictaluri	*Citrobacter amalonaticus*
Edwardsiella tarda	*Citrobacter diversus*
Edwardsiella tarda biogroup 1	*Citrobacter freundii*
Enterobacter aerogenes	*Edwardsiella hoshinae*
Enterobacter gergoviae	*Edwardsiella tarda*
Escherichia blattae	*Edwardsiella tarda* biogroup 1
Escherichia coli	*Enterobacter aerogenes*
Fusobacterium varium	*Enterobacter cloacae*
Hafnia alvei	*Enterobacter gergoviae*
Klebsiella oxytoca	*Enterobacter intermedium*
Klebsiella planticola	*Enterobacter sakazakii*
Klebsiella pneumoniae	*Escherichia blattae*
Klebsiella terrigena	*Hafnia alvei*
Kluyvera ascorbata	*Kluyvera ascorbata*
Obesumbacterium proteus biogroup 1	*Kluyvera cryocrescens*
Obesumbacterium proteus biogroup 2	*Morganella morganii*
Pseudomonas aeruginosa	*Obesumbacterium proteus* biogroup 2
Pseudomonas fluorescens	*Proteus mirabilis*
Salmonella I	*Pseudomonas aeruginosa*
Salmonella II	*Pseudomonas aureofaciens*
Salmonella III	*Pseudomonas fluorescens*
Salmonella IV	*Pseudomonas putida*
Salmonella choleraesuis	*Salmonella I*
Salmonella gallinarum	*Salmonella II*
Salmonella pullorum	*Salmonella III*
Salmonella typhi	*Salmonella IV*
Serratia fonticola	*Salmonella choleraesuis*
Serratia liquefaciens	*Salmonella paratyphi*
Serratia marcescens	*Salmonella pullorum*
Serratia odorifera	*Serratia fonticola*
Vibrio alginolyticus	*Serratia liquefaciens*
Vibrio campbellii	*Serratia marcescens*
Vibrio cholerae	*Shigella sonnei*
Vibrio harveyi	*Vibrio alginolyticus*
Vibrio parahemolyticus	*Vibrio cholerae*
Vibrio vulnificus	*Vibrio harveyi*
Yersinia ruckeri	*Vibrio parahemolyticus*

Table 2-3 cont. Bacterial species possessing lysine or ornithine decarboxylase.

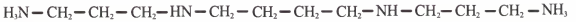
Vibrio vulnificus
Yersinia enterocolitica
Yersinia frederiksenii
Yersinia intermedia
Yersinia kristensenii
Yersinia ruckeri

Source: Taylor and Sumner, 1987, In *Seafood Quality Determination: Proceedings of an International Symposium*, D. E. Kramer and J. Liston (Eds.).

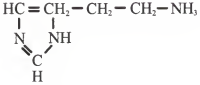
Putrescine

Cadaverine

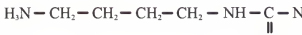
Spermidine



Spermine



Histamine



Agmatine

Figure 2-2. Chemical structure of some biogenic amines.

species and their concentrations, as well as other factors (Shahidi, 1994; Murray and Burt, 1985; Love, 1980).

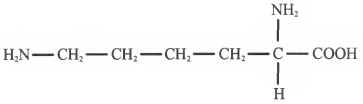
Amines embrace a wide range of NPN compounds. Some amines are produced by inherent endogenous enzymic activities which yield physiologically necessary compounds such as spermine, spermidine, histamine, etc. Many important neurotransmitters are primary and secondary amines and are derived from amino acids in simple pathways. Love (1980) stated that amines such as anserine and carnosine in fish, act as osmoregulators and protect tissues against the effects of sudden formation of lactic acid. Exogenous enzymes, on the other hand, yield putrefactive amines such as cadaverine, putrescine, histamine, etc. This latter group of amines are generally referred to as biogenic amines, and are generally formed in decomposing fish.

Fish flesh contains large quantities of low molecular weight NPN compounds (Finne, 1992; Shahidi, 1994). Autolytic changes increase the supply of these nitrogenous components, such as amino acids (Figure 2-3) (Jiang and Lee, 1985), which facilitate bacterial growth. These amino acids are converted by bacteria to amines, ammonia, aldehydes, and other end products such as hydrogen sulphide (H_2S), mercaptans, indoles, volatile bases and acids. It is these end products consumers generally perceive as indicative of putrefaction (Wheaton and Lawson, 1985; Finne, 1992).

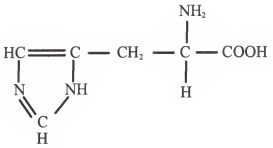
Factors Influencing Formation of Biogenic Amines

Biogenic amines in fish are, for the most part, the result of bacterial enzyme decarboxylation of free amino acids (Lambert and Moss, 1973). Draughon et al. (1987),

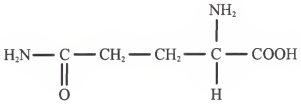
Lysine



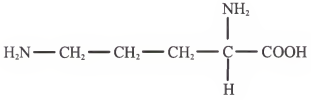
Histidine



Glutamine



Ornithine



Arginine

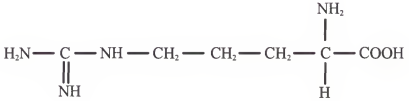
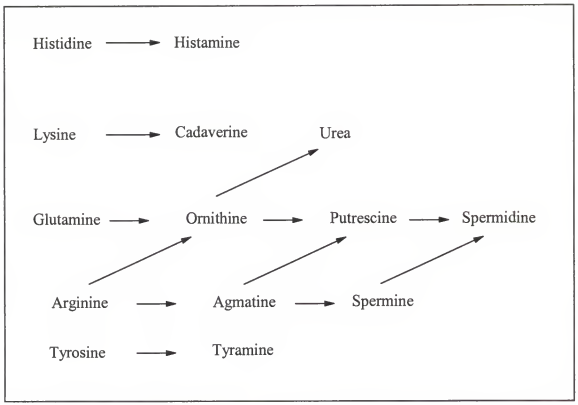


Figure 2-3. Chemical structure of some amino acid precursors of biogenic amines.

however, found that bound histidine is also utilized after the depletion of the free amino acids. The bacterial decarboxylase activity depends on pH, amino acid concentration, and temperature (Voigt and Eitenmiller, 1978). This is because each enzyme has optimal conditions (Draughon et al., 1987; Lambert and Moss, 1973) under which it is induced and functions best, provided that the relevant amino acid substrate is present in the medium (Lambert and Moss, 1973; Goldschmidt et al., 1971; Seaman, 1960).

Red muscle fish have high levels of free histidine and other free amino acids (Lukton and Olcott, 1958; Takagi et al., 1969). They provide a rich medium for decarboxylase activity, and therefore, for the formation of biogenic amines. The accumulation of biogenic amines normally occurs during the decomposition of fish. During this spoilage, bacteria decarboxylate free amino acids (Figure 2-4) in order to increase the pH of the fish medium (Eitenmiller and De Souza, 1984). Once a large population of bacteria has been established at a high (15-35°C) temperature, residual enzyme activity will continue slowly at chill refrigeration temperatures (0-7°C) even though bacterial growth ceases (Gellert et al., 1992). Liston (1990) points out that a number of psychrotrophic bacteria including Vibrios, Alteromonas and various mildly halophilic bacteria occurring on fish have been shown to produce histamine at temperatures 0-15°C. The levels of histamine produced are much lower than levels produced by mesophilic enterobacteriaceae species. However, the rate of histamine production by psychrophilics at 5°C is too slow to present a hazard for consumers.

Histidine is converted to histamine by bacterial enzyme decarboxylation (Figure 2-4). Histamine, once formed, is heat stable (Gill, 1990) and can be used as an indicator



* ⟶ indicates decarboxylase.

Figure 2-4. Schematic of biogenic amines derivation.

for fish safety. It is produced as a result of prolonged temperature abuse of fish (Liston, 1990) by only a relatively small group of spoilage organisms (Table 2-2). These mesophilic organisms proliferate in the temperature range of 22-25°C (Taylor et al., 1978). However, Van Spreekens (1987) showed that psychrophilic *Photobacterium species* were also responsible for histamine formation in mackerel and herring, and Taylor (1986) confirmed that histamine formation by psychrophilic organisms cannot be discounted.

Most biogenic amines arise from the activity of L-amino acid decarboxylases on free amino acids. The enzymes are produced by organisms such as *Morganella morganii*, *Pseudomonas, Klebsiella* and other Gram negative bacteria (Frank, 1985) (Table 2-2). Of these organisms, *Morganella morganii* is considered the most active histamine producing species, rivaled by strains of *Klebsiella pneumoniae* and *Enterobacter aerogenes* (Taylor et al., 1978). The enzymes are said to be dependent on the coenzyme pyridoxal phosphate (Lehninger et al., 1993; Zapsallis and Beck, 1986). However, Seaman (1960) stated that pyridoxal phosphate in bacterial medium has no effect on *Pseudomonas reptilivora* decarboxylation of ornithine, lysine, glutamic acid and histidine. On the other hand, glucose depresses the decarboxylase activity on those amino acids, but augments the production of arginine decarboxylase. Wei et al. (1990) demonstrated that *Hafnia's* enzymes continue to be active even under vacuum.

Most spoilage bacteria which produce decarboxylase enzymes do so at acidic pH. Gill (1990) claimed that this occurs because the organisms attempt to raise the growth medium to an alkaline pH. Yamanaka (1990) also studied polyamines in fish and found

that the pH of the muscle was initially 6.40, decreased gradually, then increased after 6 days, as microbial spoilage progressed. Hultin (1985) claimed that the ultimate pH of fish reaches a level of about 6.2-6.6, and is not affected by exercise or stress prior to death. However, some bacteria 'turn off' the production of biogenic amines when pH of the medium becomes too alkaline (pH > 7).

The optimum conditions for decarboxylase activity are not the same for bacterial growth (Eitenmiller et al., 1981). A pH of 5.8, for example, enhances the formation of cadaverine by Gram negative bacteria, while a pH of 7.0 enhances putrescine formation. Because production of putrescine begins at such a high pH, this may be one reason why putrescine levels are always lower than that of cadaverine and histamine. Yamanaka (1990) found that the lowest pH value in sardine and saury pike is at the stage of initial decomposition. He found that during decomposition the levels of histamine, cadaverine, putrescine, tyramine and tryptamine increase at different rates.

Kimata et al. (1953) demonstrated that red muscle fish distinguish themselves from white by the formation of large amounts of histamine in the red muscle fish during bacterial spoilage. The level of histamine produced is dependent on temperature and the level of free histidine (Voigt and Eitenmiller, 1978; Edmunds and Eitenmiller, 1975). Lopez-Sabater et al. (1995) suggested that proteolytic microorganisms are required to release histidine from proteins which leads to increased histamine formation. Kimata (1961) claimed that there is considerable difference in the histamine production in extractives prepared from different kinds of red muscle fish. This difference he attributed to the rate of proteolytic release of histidine from fish tissue, may help explain, in part,

the disproportional relationship between histamine formation and free histidine levels in fish tissue (Fletcher et al., 1995; Frank, 1985).

On the other hand, Yamanaka et al. (1982) found that bacteria was not responsible for histamine formation in yellowfin tuna and marlin. These fish were described as having had a bitter or pungent taste. In these cases, where bacterial activity was clearly not excessive, the presence of excessive histamine was attributed to the high activity of endogenous histidine decarboxylase, in what appeared to be abnormal meat, even though the meat was considered still fresh. Yamanaka et al. (1982) concluded that a high histidine decarboxylase activity is, therefore, important for the formation of high levels of histamine in fish.

It is evident that histidine decarboxylase from different microorganisms are not all equal in their activity (Ienistea, 1973; Eitenmiller et al., 1981). Although they may require some coenzyme/cofactor for their activity, enzymatic action is dependent on the level of free histidine available for decarboxylation (Kimata, 1961). Etkind et al. (1987) concluded that the availability and amount of free histidine, as well as the presence of histidine decarboxylase, determine the level of histamine accumulation. Only a limited number of bacterial species (Table 2-2), however, are known to produce histidine decarboxylase (Eitenmiller and De Souza, 1984), and therefore, are of importance in histamine intoxication (Frank, 1985; Taylor et al., 1978).

Amino Acids and Their Importance in Fish Quality and Spoilage

Free amino acids in fish muscle

The basic units of proteins are amino acids. These units are produced when protein is hydrolyzed using acids, alkalis or enzymes. The constituent amino acids are linked by peptide linkages into polymers resulting in peptides and proteins. Protein is the most important food component of fish (Liston et al., 1963) and is described by Nettleton (1985) as "the cardinal virtue of seafood." It is second in quantity only to water, ranging from 6-28%, and averaging 18-20% (Stansby and Olcott, 1963). Chemical and enzymatic changes during storage affect proteins which ultimately give rise to off-flavors and odors (Davidson, 1975), products of amino acid break down. The importance of amino acids in fish are well established, from the perspective of nutrition, fish meal production, and fish flavors (Stansby and Olcott, 1963; Osterhaug et al., 1963), but few studies are available on the role of amino acids in assessing fish freshness and spoilage quality.

It is known that free amino acids play a very important role in bacterial spoilage (Jay and Kontou, 1967; Ingram and Dainty, 1971), and they are the major substrates responsible for rapid microbial spoilage in fish (Jay, 1992). This microbial spoilage leads to the most extensive deteriorative changes which eventually renders the fish unmarketable (Liston et al., 1963).

Of the chemical, enzymatic, and microbial spoilage which occur in refrigerated and iced fish, microbial spoilage is considered the main mode of spoilage (Davidson, 1975). After the passage of rigor mortis, muscle fibers release juices, proteolytic activity

intensifies and bacterial proliferation occurs. The bacterial spoilage is facilitated by exogenous enzymic activity and begins soon after the fish dies. These bacterial enzymes decompose the fish tissue, resulting in a sour, grassy, or acid odor, and the flavor of the fish changes. As the changes intensify, bitter, sulfide, or ammoniacal off flavors are produced (Wheaton and Lawson, 1985).

Of the many free amino acids, histidine, lysine, and ornithine are important because they are the immediate precursors of histamine, cadaverine and putrescine (Figure 2-2) (Sikorski et al., 1990). Histamine is known to be the cause of scombroid poisoning (Arnold and Brown, 1978), and evidence shows that cadaverine and putrescine are potentiators of histaminosis (Taylor and Sumner, 1987).

Free Amino Acids in Fish Spoilage

The free amino acid profile is one of the characteristics which differs between fish species (Ranke, 1959; Bramstedt, 1962). There are always free amino acids present in the blood and flesh of fish, which are generated in the course of transportation, anabolism, and catabolism. They are derived from dietary proteins by protease activity in mucosal cells and are transported by hematogenous routes to cells. Free amino acids undergo biosynthetic and oxidative reactions in the cells of fish (Love, 1980; Zapsallis and Beck, 1986) and are in a dynamic state of degradation and synthesis. Sakaguchi and Kawai (1970a) demonstrated that, even within the same species, the free amino acid composition of fish varies with the stage of growth, age, diet, and physiological conditions. These free

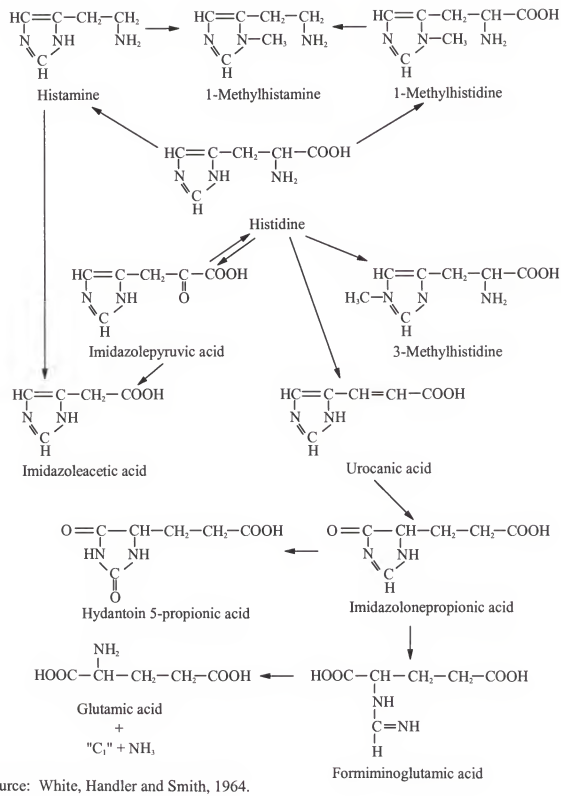
amino acids, just like polyamines, act as a buffer against sudden changes in pH for the fish (Love, 1980).

The importance of free amino acids in the role of fish spoilage is illustrated by the fact that amino acids are a major part of the substrates responsible for rapid microbial spoilage. As simple low molecular weight compounds, they provide the essential nutrients for the rapid growth of microbes after the death of a fish (Finne, 1992; Jay, 1992). Enzyme activity, specifically deaminase, breaks down amino acids to ammonia and carbonyl compounds, both of which signal freshness deterioration and spoilage of fish tissue (Schmitt and Siebert, 1961; Bramstedt, 1962; Liston, 1973). Amino acids are converted to volatile, often malodorous compounds such as ammonia, putrescine, cadaverine, hydrogen sulphide, and other sulfurous compounds generated by the break down (Ingram and Dainty, 1971; Hultin, 1984). The later breakdown of proteins contributes additional free amino acids, thus permitting additional or continued bacterial activity (Ingram and Dainty, 1971; Liston, 1973). Proteolysis, though, plays no direct part in the formation of spoilage odors (Lerke et al., 1967; Gardner and Stewart, 1966; Jay and Kontou, 1967). Consequently, the free amino acids in fish are of great importance as a contributor, not only to the flavor (Finne, 1992; McCoid et al. 1984; Jones, 1969), but also to their spoilage (Ingram and Dainty, 1971; Ashie et al., 1996).

Histidine is one of the free amino acids relatively abundant in red muscle fish (Takagi et al., 1969) and is generally kept at a constant level in the muscle (Sakaguchi and Kawai, 1970b; Nose, 1973). The large quantities of free histidine is characteristic of fatty fish (Ito, 1957; Hibiki and Simidu, 1959; Konosu et al., 1974; Stockemer, 1982),

while nonfatty fish contain little or none in the free state, despite histidine being a major component of muscle proteins. Histidine levels are generally regulated by histidine deaminase and urocanase (Sakaguchi and Kawai, 1970b), two enzymes involved in the first two steps of histidine degradation. Figure 2-5 shows several metabolic pathways of histidine. Quantitatively, decarboxylation is not a major route of amino acid metabolism in animals, nevertheless, it is important for the synthesis of certain amines such as histamine (Takagi et al., 1969).

Like histidine, lysine is one of the free amino acids present in the nitrogenous extractives of fish (Shewan and Jones, 1957). Shewan and Jones (1957) claim that the total amount of extractives remain fairly constant, although individual constituents fluctuate considerably, due to seasons and other factors. The lysine variation, they stated, may be linked with the spawning cycle and the mobilization of lysine for formation of milt and roe. In their study, Shewan and Jones (1957) observed that the change in levels of individual amino acids varied but the patterns were similar in different batches before and after spawning. Bacterial activity was reported to be responsible for the steady increase in lysine over the period of storage. Gardner and Stewart (1966), on the other hand, showed that changes in glutamine and glutamic acid were mainly due to bacterial glutaminase activity. However, Shewan and Jones (1957) reported that little change occurred in most of the free amino acids during storage at 0°C.



Source: White, Handler and Smith, 1964.

Figure 2-5. Pathways of histidine metabolism.

Free Amino Acid - A Fish Quality Index?

During chilled storage, considerable changes occur which affect proteins (Davidson, 1975). These changes provide a possible basis for investigation of the influence of spoilage on the composition of free amino acids (Ranke, 1957). Proteolytic enzymes and peptidases during the first few days post mortem result in an increase, for example, in lysine during ice storage and transportation of fish (Shewan and Jones, 1957; Bramstedt, 1962). Shewan and Jones (1957) showed that among the chemical changes that satisfy the conditions for defining a quality index, total volatile base and lysine are possible candidates. They found that in cod stored at 0°C, these two indices changed distinctly, as spoilage or storage time progressed. Jacober and Rand (1982) expressed the view that tyrosine may be an index of freshness, because it seemed to parallel the formation of total volatile base-nitrogen (TVB-N) substances. They reported that since amino acids could be metabolites occurring during autolytic changes in fish, this group of compounds might reflect quality.

Gould and Peters (1971), looking at winter flounder, concluded that amino acid analysis may not be sufficiently sensitive and specific to assess total fish quality because of variations in their levels. In addition, Liston et al. (1963) pointed out that the actual relationship between bacterial activity and chemically detectable changes in the levels of amino acids is not clear.

There is natural variation in the concentration of free amino acids present in tissues of various fish species. This inherent variation is a source of intrinsic variation of fish flavor (Thomson et al., 1980) and spoilage (Takagi et al., 1969; Takagi et al., 1971).

These variations in free amino acids levels also result in differences in concentrations of amines produced during quality deterioration. For example, arginine is a major free amino acid in fresh rock lobster and squid, and the product of arginine decarboxylation (Figure 2-2) is agmatine, the chief biogenic amine in these species (Yamanaka, 1990). On the other hand, histidine is a major free amino acid in scombroid fish and histamine is a major biogenic amine produced during spoilage. Ehira and Uchiyama (1986) pointed out that there are differences in freshness deterioration which varies from species to species. These differences may well be, in part, because of variations in levels of free amino acids between species.

There is ongoing research seeking better indicators of the incipient stage of fish spoilage, and/or the degree of freshness. This is due to the Food and Drug Administration's regulations and the implementation of hazard analysis critical control points (HACCP) (FDA, 1995b); the need to assure consumers of fresh and safe seafood products; the need for standardized analytical methods for objective quality determination; and the need to enhance processing, distribution, and storage systems (Ehira and Uchiyama, 1986; Luten et al., 1992). Consequently, an understanding of the levels of free amino acids in fish helps explain biogenic amine formation (Kirschbaum et al., 1994) during fish decomposition, and may be an important biochemical clue to the freshness quality of fish (Shewan and Jones, 1957; Bramstedt, 1962).

Methods for amino acid analysis, however, require extensive preparatory work (Lindroth and Mopper, 1979) and often involve the use of picric or trichloroacetic acid (TCA) as extracting solvents (Sakaguchi et al., 1982; Konosu et al., 1974). Given the

importance of free amino acids in fish deterioration, their role as precursors of biogenic amines, and the inability of our method to separate high levels of histidine from low levels of glutamine, it became necessary to develop a method that enabled the rapid analysis of glutamine, histidine, ornithine and lysine in fish. The first objective, however, was to investigate the development of histamine, cadaverine and putrescine, specific biogenic amines associated with the spoilage of red muscle fish, using mahi-mahi as our model. Our second objective was to correlate the amines with their precursor amino acid levels, TVB-N levels, and sensory evaluations of the quality of the refrigerated fish.

Fish Freshness and Spoilage Quality

The Concept of Quality

The concept of quality is not always well defined. Kramer (1966) reminds us that this term can refer to good or poor, and that the term quality is not necessarily that which is excellent or highly desirable. Kramer (1966) states that the categories of quality include quantitative and sensory factors. It is, therefore, understandable that consumers perception of fish quality differs from that of experts and food scientists. To consumers, quality is very important, and generally refers to the sensory qualities of taste, odor, texture and appearance (Slavin, 1987). To scientists, these sensory criteria are subjective, thus, objective or quantitative means are used to determine and describe these qualities. These objective measures include attributes such as wholesomeness, which ordinarily cannot be evaluated by consumers (Kramer, 1966). No agreement exists, however, on the

use of any one test as acceptable for describing seafood quality (Jacober and Rand, 1982). Herein lies the difference between consumers and experts: consumers may view certain characteristics as important, while experts may not give consideration to these same characteristics (Bisogni et al., 1987).

The Concepts of Freshness and Bacterial Spoilage

Botta (1995) stated that the term "freshness" refers to a concept rather than a distinct object in as much as different people have various definitions for the term. This freshness, Sikorski et al. (1990) stated, involves the sensory assessment of post mortem changes in fish and shellfish. Ehira and Uchiyama (1986) pointed out that experimental results indicated that the initial loss of freshness in fish is not caused by bacterial action, but from biochemical changes in muscle tissue. Farn and Sims (1986) also concluded that there are other spoilage patterns besides bacterial. Hence biochemical freshness is different from that of bacterial spoilage.

After the death of a fish, endogenous enzymes remain active and are involved in biochemical changes during the initial stages and days of storage (Ranke, 1959) before bacterial spoilage becomes significant. These biochemical changes lead to flavor and odor changes during the initial stages and days of storage. Ehira and Uchiyama (1986) confirm that fish proteolytic enzymes and peptidases play important roles in the biochemical spoilage of fish during the first few days post mortem. Gill et al. (1987) stated that, in tuna, tissue softening also occurs in the absence of putrefaction.

Jay (1992), referring to the onset of bacterial spoilage, concluded that incipient spoilage is not easily determined by current methods. As such, maintaining a low temperature is generally the most important factor used to control fish quality degradation. To control low temperature spoilage of fish, in the words of Davidson (1975) "keep it cold; keep it clean; keep it moving!".

Castell and Anderson (1948) pointed out that during fish spoilage bacterial cultures do not produce just one typical odor. Instead, usually a succession of two or more distinct different odors are produced, each blending into the previous as spoilage progresses. In this discussion the term freshness refers to the sensory criteria of appearance, odor and flavor. Of these attributes, odor, appearance and flavor are the most important criteria used by consumers to determine fish freshness (Stansby, 1963). These characteristics, like texture, can also be assessed in the laboratory by scientists.

Many chemical tests have been proposed as useful measures of freshness and spoilage of fish. These tests include TVB-N, biogenic amines, ammonia, K-value, etc. None of these, however, is universally applicable. These tests are expected to correlate with various sensory conditions such as flavor and odor. However, there are differences in the mode of spoilage due to the nature of fish tissue composition (Gill, 1990). Fatty fish (5-23%) may undergo extensive enzymatic and oxidative rancidity prior to the onset of bacterial decomposition. Unlike fatty fish, proliferation of bacterial activity predominates at the onset of decomposition in lean fish (fish with less than 2% lipids in the edible tissues) (Woyewoda, 1990). Similarly, the composition and amount of free

amino acids would influence the types of amines which are produced, since the content of free amino acids in fish is responsible for its quick bacterial spoilage (Ranke, 1959).

Stansby (1963) points out that freshness is dependent on the presence or absence of many chemicals. Hence, a group test involving the determination of different substances, e.g. TVB-N, tends to be more reliable than a specific test such as TMA, or ammonia. Sikorski et al. (1990) also state that no single indicator substance is sufficient to qualify seafood as fresh.

Hazard Analysis Critical Control Points

HACCP is a hazard prevention system designed for assuring production of safe seafood products (FDA, 1994). Its enforcement became mandatory in December of 1997. HACCP applies technical and scientific principles in the production of seafood products sold commercially in the United States and exported abroad. Thus, it affects importers as well as processors and exporters of fish. Rather than depend on inspection, the system seeks to prevent hazardous problems which may occur during the processing of foods, beginning from the point of raw material production (FDA, 1994). HACCP, therefore, is a methodical and systematic application of appropriate means to safely produce wholesome foods (Stevenson, 1995), coupled with FDA inspections based on the HACCP system. Since its inception, many producers and distributors have expressed satisfaction with HACCP (Curlee, 1997).

To accomplish its preventative goal, HACCP identifies potential food safety hazards, i.e. intrinsic and extrinsic biological, physical and chemical, and sanitation, in

the processing place. This systematic approach encapsulates the following seven basic principles (FDA, 1994):

1. Conduct a hazard analysis so as to identify where the significant hazards can occur. By preparing a list of the steps involved in the process, the potential points of hazards are identified and preventive measures described.

2. Identify the steps in the manufacturing process that may be critical control points (CCPs).
   These steps include chilling, cooking, and sanitation procedures, both employee and environmental. For example, the guidelines suggest that the internal temperature of freshly harvested fish be brought to 10°C or below within 6 hours of death, and quickly brought towards the freezing point to prevent longer-term low temperature spoilage.

3. Establish critical limits for the preventive measures associated with each identified CCP.
   Critical limits in temperature, time, pH, sensory information, etc., are set for each CCP.

4. Establish CCP monitoring requirements.
   This entails the establishment of a planned sequence of observations or measurements to assess adherence to operational parameters of CCPs, and for recording and using the results of monitoring to adjust the process and maintain control.

5. Establish corrective actions to be taken when monitoring.
   This is necessary because of imperfections such as deviations from established critical limits.

6. Establish effective record-keeping procedures that document the HACCP system.
   This step requires the preparation and maintenance of a written HACCP plan that sets out the hazards, CCPs, critical limits, monitoring, record keeping and other procedures.

7. Establish procedures for verification that the HACCP system is working correctly.
   This procedure verifies that critical limits are adequate to control hazards, ensure the proper working of HACCP plan, and the documentation of periodic evaluation of the plan and its implementation.

Gill and Kobayashi (1990) pointed out that discussion of seafood quality cannot be complete without the consideration of safety. Since the introduction of HACCP, the food safety emphasis has shifted from inspection and reaction to outbreaks, to prevention of outbreaks (Stevenson, 1995). HACCP applies to all stages of seafood production and is relevant to the seafood industry because of frequently reported seafood related illnesses (Gellert et al., 1992; FDA, 1994) such as: water-borne viruses in molluscan shellfish which are usually consumed raw or partially cooked, ciguatera toxin found in warm water fish, and scombroid poisoning resulting from time/temperature abuse of fish (Appendix B and C). The alternatives to HACCP are not as efficient and cost effective. One alternative requires continuous visual inspections (which includes reliance on end-product testing and large sample sizes), and by which few hazards associated with seafood are detected. Another alternative is to direct additional resources toward increasing the frequency of FDA's inspection of seafood (FDA, 1994).

Fish Quality Indicators

Biogenic Amine Indicators

Among the criteria for a suitable objective quality test, Gill (1990) lists the measurement of a compound, absent or present, in constant amounts in living tissue. This compound must either increase or decrease proportionally with quality change, and the results should not be affected by the process of analysis per se. Thus, careful and detailed observation of one or more of these biogenic amines probably could be used as an

indicator of spoilage and the degree of freshness for one species, or maybe a family of fish (Mietz and Karmas, 1978; Taylor and Sumner, 1987; Yamanaka, 1990).

Castell (1971) pointed out that at least two factors, or quality tests, are essential for the determination of fish quality: microbial and physiological. A fish's physiological condition is affected by season, as well as by environmental and other influences which affect chemical composition, texture, odor, and microbial activity (Castell, 1971). Thus the variations in the physiological conditions of fish make it difficult and challenging to obtain a single biochemical indicator of fish quality.

Jay (1992) claims that the most reliable indicator of quality tends to be product specific. Therefore, which of the biogenic amine(s) to use as an indicator, would depend on the unique free amino acid content of the fish species, the muscle type used for analysis, and the microflora present on the fish. However, owing to the heterogeneity of fish and shellfish species, several authors claim that no one biogenic amine would necessarily serve as a useful indicator of freshness quality in all the different species of fish (Mietz and Karmas, 1978; Yamanaka, 1990; Jay, 1992).

There is evidence that biogenic amines are useful indicators of incipient fish spoilage and the degree of freshness deterioration (Mietz and Karmas, 1978; Yamanaka, 1990; Gill, 1990; Ohashi et al., 1991; Botta, 1994). Their absence, though, does not necessarily reflect wholesomeness and high product quality (Gill et al., 1987; Farn and Sims, 1986). Farn and Sims (1986) showed that cadaverine, putrescine, and other putrefactive amines are at low levels in the initial stage of storage but their formation increases at the latter stages of decomposition.

Yamanaka (1990) stated that polyamines contribute to putrid odors indicative of decomposition. He showed that levels less than 15 mg/100g of cadaverine in sardine, saury pike and squid were acceptable. However, levels greater than 20 mg/100g indicated advanced decomposition. The initial decomposition of the fish showed levels between 15 to 20 mg/100g of cadaverine, which correlated well with sensory evaluations (Yamanaka, 1990). Fresh saury pike had no histamine and was not detected until the initial stage of decomposition. On the other hand, polyamines were found to be absent in fresh sardine muscle but histamine was present at 8.5 mg/100g.

At the higher temperature of 20°C, decomposition was more rapid but the pattern was the same for both sardine and saury pike (Yamanaka, 1990). In sardines, histamine was reported to be at toxic levels (104 mg/100g) only after one day of storage at 20°C, although the muscle was acceptable. This illustrates the potential danger of histamine being present in seemingly fresh fish, and underlines the need for an informative indicator that can quickly be determined and will correlate with the degree of spoilage and amount of histamine present in fish. A similar pattern was observed for the histamine in saury pike muscle at 5°C and 20°C. However, histamine was not present at the beginning and was formed only at the initial stage of decomposition. The relative levels of histamine in the two fish were different. Histamine formation, therefore, may be different in different species, and its levels may differ at the same stage of storage and decomposition even for the same species (Fletcher et al., 1995).

Microbial decarboxylation of free amino acids results in production of biogenic amines which are considered indicators of fish safety and spoilage (Mietz and Karmas,

1978; Finne, 1992). Mietz and Karmas (1978) looked at biogenic amines as a spoilage index for rockfish, salmon, lobster and shrimp. Their work showed good correlation of cadaverine, putrescine, spermine and spermidine with sensory scores, resulting in the formulation of a spoilage index. Mietz and Karmas (1978) demonstrated that their polyamine index was indicative of decomposition, particularly cadaverine and putrescine. They reported the general loss of spermine and spermidine as decomposition progressed. Kim and Bjeldanes (1979) also found that spermidine levels decreased in canned spoiled tuna but spermine levels were the same in both spoiled and unspoiled samples. Ritchie and MacKie (1980), working with mackerel and herring in storage, reported an initial decrease of spermidine and spermine, followed by an increase. Effects of species, season, temperature, etc. may be responsible for such differences.

Nakamura et al. (1979) suggested that the differences in the level of bacterial flora may be responsible, in part, for the differences in the level of polyamines found in food. Van Spreekens (1987) showed that psychrophiles (*Photobacterium spp.*) were also responsible for histamine formation in mackerel and herring. However, these microbes required at least 0.5% NaCl in the medium and were thermolabile. Van Spreekens (1987) suggested that minor levels of histamine in fish, with high levels of free histidine, may be indicative of potential intoxication or a lack of wholesomeness.

The ability to produce putrescine and cadaverine is widespread (Table 2-3), especially among the Enterobacteriaceae (Taylor and Sumner, 1987). Taylor and Sumner (1987) pointed out that many bacterial species possess ornithine and/or lysine decarboxylase(s). They suggested that potentiators of histamine toxicity (putrescine and

cadaverine) may be formed in fish by bacterial species not involved in histamine formation, and that their presence could result in the hazardous effects of relatively small amounts of histamine (Arnold and Brown, 1978; Hultin, 1985).

It is evident that the levels of histamine, putrescine, and cadaverine in spoiling fish, vary, depending on the spoilage bacteria, type of fish, and storage conditions (Taylor and Sumner 1987). Cadaverine levels may reach 10 to 60 mg/100g, and histamine may be up to 500 mg/100g. Putrescine may be up to 10 mg/100g, but is usually less. Taylor and Sumner (1987) claimed this is because the quantity of ornithine, the direct precursor of putrescine, may be limited in fish tissue. Putrescine, however, is the precursor for the synthesis of spermidine and spermine (Figure 2-2) which may be a possible reason for very low levels of putrescine, in light of the level of its precursor - ornithine. Alternatively, it may be possible that putrescine is slowly derived from the break down of spermidine during fish spoilage. This lack of clarity illustrates the need for more detailed biochemical study of fish quality deterioration. Kimata (1961), however, concluded that there is no direct relationship between degree of freshness or spoilage and the amount of histamine. Consequently, the use of histamine as a chemical index of fish decomposition has long been considered misleading (Walters, 1984).

It is reasonable, therefore, to postulate that the concentrations of ornithine, and its product putrescine, may correlate with the stage of bacterial deterioration of fish. The nature of this correlation is not known, nor has it been determined. It is known, though, that putrescine, cadaverine, histamine, spermidine, and spermine have been used as a spoilage index (Mietz and Karmas, 1978). Cadaverine and/or putrescine levels, on the

other hand, may indicate the incipience of fish freshness deterioration, since they, along with TVB-N, are formed during spoilage. From our observations, cadaverine occurs at low levels even in fresh fish, unlike TVB-N. It seems therefore, that cadaverine and putrescine may be used to indicate the degree of decomposition (Rogers and Staruszkiewicz, 1997). Alternatively, cadaverine and putrescine may very well correlate with levels of histamine, and/or sensory hedonic evaluation, TVB-N, and/or amino acid levels.

Yamanaka (1990) stated that cadaverine agrees well with the degree of decomposition in particular species, and within set storage temperatures. He observed that cadaverine formation is about 3-4 times greater than putrescine, and recommended cadaverine as a useful index for fish spoilage, while agmatine should be used exclusively for squid. Levels of putrescine and cadaverine increase during decomposition, with the highest concentration levels occurring at advanced decomposition, which then tends to decrease with prolonged storage. Yamanaka (1990) further added that histamine is not always useful as an indicator of fish decomposition. Therefore, it is hypothesized that together, putrescine and cadaverine, may be sufficient as a useful index of fish safety and decomposition. These biogenic amines probably correlate with TVB-N, one or more of the precursor amino acids, and/or sensory scores.

More recently, Duflos et al. (1999) reported the correlation of levels of biogenic amines with a freshness index. Sims et al. (1992) reported good correlation between expert sensory evaluations and levels of putrescine and cadaverine in canned skipjack tuna. It is evident that some sort of correlation exists between some biogenic amines and

fish freshness quality. The issue, however, is which of these amines provide(s) the best possible indication of quality for which particular fish species and whether or not it (they) will be a good indicator of the likeliness of toxicological levels of histamine.

Total Volatile Base-Nitrogen

Total volatile base (TVB), expressed as nitrogen (TVB-N), includes trimethylamine (TMA) and ammonia (Gill, 1990). TVB-N is measured by either of three techniques: potentiometric end point titration detected either with a pH electrode or color indicator; colorimetry test used when very low amounts of the TVB-N compounds are present; and ammonia specific electrode (Brinkmann Instruments, Inc., Westbury, NY).

Shewan et al. (1971) stated that formation of volatile bases is one of the most characteristic features of fish spoilage. These volatile bases mainly include ammonia and lower amines such as tri- and dimethylamine. Antonacopoulos (1971) recommended that TVB-N should be used in conjunction with sensory panel assessment, because standards of acceptability cannot be based on objective methods only. Despite its limitations, TVB-N is widely employed in the assessment of fish quality by industry and regulatory authorities (Del Valle et al., 1984; Vyncke et al., 1987). Gill (1990) showed that TVB-N increased with the degree of squid spoilage, although microbial quality remained constant, indicating that spoilage is predominantly due to enzymic activity. Ammonia is a volatile component of spoiling tissue. Post mortem production of ammonia is a function of storage condition, storage time, and results from several enzymic processes including: deamination of free amino acids, degradation of nucleotides, and oxidation of

amines. On the other hand, TMA is a direct result of bacterial activity, but its correlation with bacteria number, in some cases, is poor (Gill, 1990). Thus, while TVB-N, TMA and NH_3 are used to measure the noticeable stages of fish deterioration, they do not identify the incipient stages of freshness quality deterioration (Botta, 1995). Consequently, no one objective criteria may suffice for all forms of quality determination, and the criteria used must also compare well with subjective evaluations.

Wekell et al. (1987) observed a good correlation between TVB-N and sensory analysis of surimi based fabricated seafood products. Hong et al. (1996) reported a parallel increase in TVB-N and aerobic plate count during storage of mackerel under modified atmosphere packaging. They suggested that TVB-N may be useful as a quality indicator for mackerel fillets under MAP. Aubourg et al. (1997) observed a good correlation ® = 0.93) between TVB-N and quality measurements of fluorescent lipid compounds for sardines during storage at 0° and 15°C. Bennour et al. (1991) found that for mackerel stored in ice, TVB-N was 23 mg/100g at the time of rejection based on sensory analysis. This level of TVB-N corresponded to total plate count of $1x10^6$ CFU/g. El Marrakchi et al. (1990) found that TVB-N accurately predicted the spoilage process of sardines during storage in ice. Hollingworth et al. (1991) reported contrasting results of TVB-N as an effective chemical indicator for vacuum packed, pasteurized flaked imitation crabmeat.

Nunes et al. (1992), on the other hand, concluded that TVB-N was not a good freshness indicator for sardines stored in ice. Kolakowski (1986) concluded that volatile bases cannot be regarded as a freshness criteria for Antarctic krill, because of

considerable variation in the TVB-N content. Licciardello et al. (1984) observed that gamma radiation of spiny dogfish fillets (*Squalus acanthias*) suppressed formation of volatile bases (TVB-N). Therefore, TVB-N will not be a good indicator for assessing spoilage in radio-pasteurized dogfish, nor several other species, based on the fish species and storage conditions.

It is evident that TVB-N results are influenced by the fish sample itself (Vyncke et al., 1987). These authors, in a collaborative study, reported important systemic errors among participating laboratories. They thought that the method itself is either inadequately described, or very sensitive to differing environments in the various laboratories. Horwitz et al. (1998) suggest a simple method which helps evaluate data from such interlaboratory studies. Vyncke et al. (1987) used the analytical technique proposed by Codex Alimentarius (FAO) as the common method versus the methods normally used by the respective laboratories. The common method used 7.5% trichloroacetic acid for extraction of TVB-N compounds, and a micro-Kjeldahl distillation apparatus. It is necessary to determine for which species of fish TVB-N would be a good indicator, and which methods are best suited and easily applied. To this end, much work and data gathering will need to be done.

Sensory Evaluation

Sensory evaluation continues to be the main mode of fish quality assessment, especially where gas chromatographic methods are ineffective and do not identify the causative compounds responsible for off-flavors (Bett and Dionigi, 1997). Sensory

evaluation of fish quality may be subjective because it is dependent on the human senses, as is often used by consumers in the market place, to distinguish differences in quality (Wheaton and Lawson, 1985). This method makes use of the senses of sight, touch, smell and taste to examine the parameters of appearance (color, gaping, firmness, mushiness, dryness), odor and flavor (Wheaton and Lawson, 1985; Gill, 1990; Mennella, 1998). The method is generally descriptive, as described by Wheaton and Lawson (1985) in their list of sensory criteria of fish quality, and can be very expensive in terms of maintaining trained personnel (Bett and Dionigi, 1997).

Sensory evaluation can also be objective when used by scientists and trained panels but it has a number of limitations (Hollingworth et al., 1991; Bett and Dionigi, 1997; Stone and Sidel, 1998). The human senses are often used objectively for grading fish. Woyewoda et al. (1986) describe the step-by-step procedures generally used by experts for the objective grading of fish. However, with the advent of computers, newer physical, chemical, and microbial methods are being developed, all of which seek to either replace or complement sensory measurements of fish quality with inexpensive objective techniques (Gill, 1990; Bomio, 1998; Infometrix, 1999). Indeed, the difficulties of time and effort in the training and running of sensory panels, and the reliance on specific personnel, have also led to the search for more convenient methods of nonsensory assessment (Hanna, 1992). However, Hollingworth et al. (1991) reported that sensory evaluation, despite its limitations, remains a reliable quality assessment method.

AromaScan

The AromaScan A32S/8S is a tool designed to simulate the sense of smell. It complements the work of sensory analysts and brings consistency and objectivity to the field of sensory analysis (AromaScan plc, Crewe, U.K.). The human nose senses volatile compounds which stimulates the olfactory region, resulting in messages being sent to the brain, enabling recognition of various aromas (Mennella, 1998). The AromaScan, like the human sense of smell, detects the presence of volatile chemicals and records the characteristic and intensity of these aromas. The instrument then generates a pattern of the results obtained from the samples used. These results can then be processed using statistical tools built into its software to produce an easily interpreted mapping (Sammon map) of the results. The software also allows use of standard results for training of the instrument to build an artificial neural network (ANN), or for further statistical analysis. The instrument has been successfully used for assessment of several products including honey, orange juice and fish (AromaScan plc, Crewe, U.K.), and for control of cork stopper quality used in bottling wines (Rocha et al., 1998; Rohrback, 1996).

The AromaScan A32S/8S is comprised of an analyzer unit (A32S) and a sample station (A8S). The analyzer contains an array of 32 sensors made of polymers which change their electrical resistance when volatile compounds adsorb to them. Each sensor responds differently to the same compound, giving each a different characteristic response (AromaScan plc). The sample station generates reference air of known quality and humidity used for flushing a polyester/polyethylene pouch containing the sample.

This pouch is then conditioned in a temperature controlled chamber (4-35°C) until the head space is equilibrated. The equilibrated head space is then pumped into the analyzer across the sensor array where each sensor gives a relative response to the range of volatiles given off by the sample. This last step is the data acquisition phase.

The mapping tool displays the results in a two- or three-dimensional plot in which differences in samples become apparent. The ANN performs a pattern recognition based on comparisons of incoming data with the database previously developed during the training of the machine. For training, standard samples of known qualities are used (Rocha et al., 1998). To attain this end, it requires several measurements to categorize samples for quality control purposes. It is the instrument's software capabilities that make the AromaScan a good quality control tool.

The AromaScan makes use of chemometrics, or pattern recognition, to classify samples into categories. Chemometrics is a statistical approach to the interpretation of patterns in multivariate data (Rohrback, 1996). It generates faster and more precise assessment of sensory properties, as well as food composition and physical properties.

The Electronic Nose and Hewlett Packard Chemical Sensor HP 4404 are other examples of techniques relying on chemometrics (Infometrix, Inc., 1999). These instruments vary based on the number of sensors, their structure and configuration, and capabilities. However, they all require a thermally equilibrated sample vapor and generate a pattern of responses for the samples analyzed (Infometrix, 1996). The HP 4404 uses a mass selective detector (MSD) which gives it the ability to identify the compounds that cause samples to be different (Meng and Wylie, 1999). The electronic

nose, unlike the AromaScan, uses 16 sensors. These instruments lack the specificity for odor evaluation like GC and mid-IR and make use of chemometrics whose patterns have no chemical meaning (Infometrix, Inc., 1999). Bomio (1998) points out that even if these techniques impact on sensory analysis, there will never be any technological substitute for measurement of consumers' preference and acceptance.

Microbial

Microbial methods are generally used to indicate the presence or absence of microbes in a fixed quantity of product, or to measure the total numbers of organisms. One of these methods is the standard aerobic plate count which gives a measure of the degree of contamination (Wheaton and Lawson, 1985). However, microbiological examinations may search for specific pathogens which may require the use of specialized media, e.g Niven's medium used for identification of histamine producing bacteria (Niven et at., 1981). Hanna (1992) claims that microbial enumeration methods correlate well with sensory scores of fish during the later stages of storage.

Microbial assays, however, suffer the disadvantage of the inevitable delay in the availability of results because of the growth time needed for bacterial colonies to show up on media. Hanna (1992) claims that techniques to extract organisms from samples and to cultivate them, using incubation temperatures to give results as fast as possible, give rise to varying degrees of estimation. Besides, non spoilers and spoilers are generally treated in the same way. It is problems like these that stimulate the drive for more reliable and rapid methods of microbial analysis.

Other Chemical Indicators

Wheaton and Lawson (1985) list five broad groups of methods used for fish quality assessment: sensory and microbial (discussed above), chemical, physical and statistical. Of these factors, microbial is the primary means of assessing fish quality because of the ubiquity of potentially life threatening pathogens in our environment (FDA, 1994). The millions of cases of foodborne diseases which occur each year in the United States (Flowers, 1988; Stevenson, 1995), and changing consumer lifestyles which result in demand for freshly prepared foods, are additional reasons for microbial concerns. Currently, more and newer generations of refrigerated and fresh products are reaching the marketplace to satisfy consumer demands (Farber, 1989; Merritt, 1990). These foods have lead to the potential growth of psychrotrophic pathogens. By implementation of effective control measures such as refrigeration, freezing, packaging, irradiation, etc., the potential hazards are limited.

Commonly used chemical assessment methods include the amines already discussed above, hypoxanthine (Hx), nucleotides, ammonia and TMA - the major components of TVB-N discussed above - (Jahns and Rand, 1977), and the indole test for shellfish (AOAC Official Methods of Analysis, 1990; Boee et al., 1982). Woyewoda (1990) lists ethanol, dimethylamine (DMA), formaldehyde and free fatty acids (FFA) as chemical indicators of fish quality. These last four indicators are generally the result of enzymatic activity in fish tissue during storage.

Nucleotide degradation (measured as Hx) results in the accumulation of inosine, hypoxanthine and other products during the storage of fish (Hanna, 1992). Hypoxanthine is a result of autolytic and bacterial activity. Considerable variations, however, occur in the rate of nucleotide breakdown between fish species, due to inherent variations in the intrinsic enzymes, their activity, and nucleotide levels.

The 'K' value, which is a ratio of the sum of inosine and Hx to the total ATP-related compounds, expressed as a percentage, has also been used as a fish quality index (Ehira and Uchiyama, 1986). Perez-Vilarreal and Pozo (1990) found that the K value and sensory assessment had the highest correlation with storage time of albacore in ice.

Tennyson (1999) reported on the development of a collaborative study involving the use of volatile base determination using an ammonia ion-selective electrode. This method, described by Pivarnik et al. (1998), and recommended to the Association of Official Analytical Chemists (AOAC) International Methods Board for adoption, uses ammonium chloride as a standard. Recovery efficiencies range from 88.6 to128% based on fortified fish samples, whether fresh, borderline, or spoiled. This method has a correlation, $r = 0.88$, with the steam distillation method for TVB-N determination. The electrode is claimed to have the potential to serve as an on-site rapid screening for TVB-N in fish.

Jahns and Rand (1977) described the use of an enzymatic method to assess marine fish quality. They showed that diamine analysis, using diamine oxidase reaction, has potential value as a spoilage indicator for red hake (*Urophycis chuss*) and winter flounder. They graphically showed the interaction, or pattern, of hypoxanthine and

diamine development in fish. Hall et al. (1999) described a solid-phase enzymatic assay for cadaverine and putrescine in tuna. This dipstick, or test strip, couples an amine oxidase to a peroxidase/dye system.

Hollingworth and Throm (1982) described the use of ethanol as a fish quality indicator. Woyewoda (1990) also listed ethanol as a chemical indicator of fish quality, and Hong et al. (1996) reported a linear increase in ethanol during the storage of mackerel under modified atmosphere. Other chemical indicators are peroxides, aldehydes and carbonyls which develop as a result of chemical spoilage or oxidation, and require specific laboratory equipment for determination.

Physical

Examples of physical tools used for fish quality assessment are the AromaScan, described above, the Electronic Nose, and the Torrymeter. Whereas the AromaScan and electric nose are proving to be reliable physical methods, the Torrymeter has proven to be unreliable. The Torrymeter measures the degree of freshness based on the electrical properties of the skin and underlying tissue. However, for frozen fish, thawed fish, and fish stored under varied conditions, it does not provide satisfactory readings (Hanna, 1992; Wheaton and Lawson, 1985).

It is the availability of these and other chemical and physical tools which will ultimately reassure seafood consumers, of whom Gorga and Ronsivalli (1982) said, "lack the single most important impediment to the growth of seafood consumption, assurance of quality."

Statistical

Statistical methods are used largely for the quality control of processes (Wheaton and Lawson, 1985). These methods often require determination of the correct number, size, or sample required, for example, to test the freshness of a batch of fish, or adherence to specified processing parameters such as cooking and chilling. A statistical method may very well be suitably developed for histamine assessment, because of its uneven development in fish, and the importance of histamine as a safety and quality indicator in fish and fish products. Whereas chemical tests are effective means of histamine detection and quantification, the validity of such tests depends on the statistical designs and sampling plans.

CHAPTER 3
MATERIALS AND METHODS

Treatment of Fish

Fish Procurement

Six mahi-mahi (*Coryphaena hippurus*) fish, 9-11.5 Kg each, were obtained from SavOn Seafood, St. Petersburg, FL. The fish were caught with handline in the Pacific Ocean, off the coast of Equador, in winter of 1999. They were gutted and headed on the deck and held in ice until the boat's return to the dock in the afternoon. The fish were held in ice storage throughout its delivery to St. Petersburg and to the University of Florida, Gainesville. The total product age prior to pick up in St. Petersburg was 40 hours post harvest, plus 20 hours prior to experimental treatment.

Fish Preparation and Storage

On the day the fish arrived in Gainesville, they were randomly numbered 1 to 6 and filleted. One fillet from each fish was put into the uninoculated group, and the other into the inoculated group as shown in the design diagram (Figure 3-1) below. Each fillet was cut into seven portions, 350 to 450 g each, plus the end portions, which were not used.

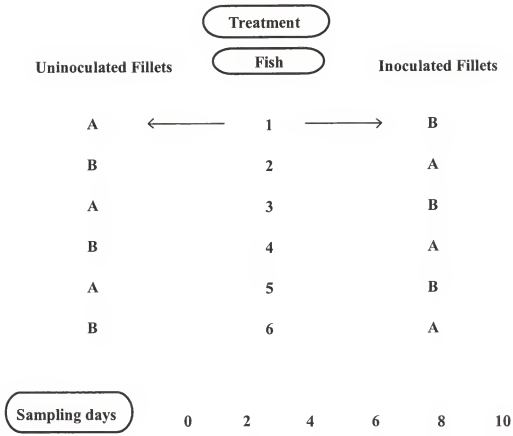


Figure 3-1. Illustration of experimental treatment design.

On the morning of day 0 (the next day), the fillets of inoculated group were separately dipped for two minutes in a prepared microbial inoculum of *Morganella morganii*. The inoculum was divided into six portions, such that each portion was equal to twice the weight of the corresponding fillet dipped into it. The fish were quickly drained and placed in labeled containers for storage. The two groups of fish were then stored at 7°C for the next 10 days.

The *Morganella morganii* culture was described by the American Type Culture Collection (ATCC, Rockville, MD) as originating from a food poisoning source and recommended a growth temperature of 37°C. The freeze dried bacteria was resuscitated in nutrient broth according to the procedures described by ATCC (1989). The culture was then plated out to ensure a monoculture was obtained and to isolate individual colonies for further work. A 24 hr broth culture was prepared using trypticase soy broth fortified with 2.0% histidine (TSBH) and of pH 6.3 (Taylor and Woychik, 1982). The composition of the broth is shown in Table 3-1. The broth was inoculated with one isolated colony from the plated resuscitated bacterial culture, and incubated for 24 hours. Immediately following the 24 hr culture, an 18 hr culture was prepared and diluted to make the inoculum. The 18 hr broth culture (10^9 CFU/mL) was diluted in sterile Butterfield's phosphate buffer of pH 7.2, yielding an inoculum of 10^5 CFU/mL.

Table 3-1. Trypticase soy broth-histidine composition.

Component	% (w/v)
Trypticase soy broth	3
Histidine	2
pH adjustment	6.3

Source: Taylor and Woychik, 1982.

Analyses

Reagents. Analytical grade boric, sulphuric and hydrochloric (HCl) acids, sodium phosphate monobasic (NaH_2PO_4), potassium acid phthalate, sodium tetraborate decahydrate, sodium hydroxide (NaOH), magnesium oxide (MgO), screened methyl red and phenolphthalein indicators, and anti-bumping beads were bought from Fisher Scientific (Fair lawn, NJ). HPLC grade methanol, ethyl acetate, toluene, hexane and filter paper were also purchased from Fisher Scientific. Amino acids and biogenic amines standards, o-phthaldialdehyde (OPA), pentafluoropropionic anhydride (PFPA), and anti-foam silicon preparation concentrate A were bought from Sigma Chemical Company (St. Louis, MO). Tetrahydrofuran chromAR and 2- mercaptoethanol were bought from Mallinckrodt Specialty Chemicals Company (Paris, KY). Ethyl alcohol-200 proof was purchased from Aaper Alcohol and Chemical Company (Shelbyville, KY). Distilled deionized water was obtained from a Photronix Reagent Grade Water System (Photronix Corp., Medway, MA).

Sampling Procedures

On each day of sampling (0, 2, 4, 6, 8, and 10) one portion of fish was taken from each fillet and placed in separate labeled ziplock sampling bags. The samples were held in ice for immediate analysis, or at -20°C until further analysis. The bags were labeled with computer generated random three-digit numbers. Frozen samples were quickly thawed prior to analysis and care was taken not to allow drip loss.

Sensory Evaluation

Seventeen experienced panelists (people familiar with sensory evaluation and who had participated in recent sensory evaluations of fish) were trained for the sensory evaluation (Appendix D and E) of the fish in this experiment. Only twelve of the panelists' scores were used for analysis because they completed the evaluations. Panelists were informed of the objective and what they were required to do. Three training sessions were held during which the panel was given specimens of very fresh and spoiled fish, and were also asked to evaluate samples given to them. The panel also discussed and decided on the category scale of 1-10. During the evaluations panelists were asked to assess the fish in each labeled pouch and to score the four attributes: 1. color (very bright, bright, dull, brown); 2. gaping (none, slight, moderate, excessive); 3. texture (very firm, firm, soft, very soft); and 4. odor (very fresh, slightly fishy, moderately fishy, ammoniacal) (Appendix E). A category scale of 1 to 10 was used for scoring, where 9-10 indicated a very good rating, and 1-2 indicated a very poor rating. All samples were clearly coded with computer generated random three digit numbers. The sensory data was then examined for correlation with TVB-N values, biogenic amines levels, amino acid concentrations and microbial numbers.

Sensory test, AromaScan mapping, and microbial tests were carried out on the same day of sample removal from the treatment conditions. TVB-N assays preceded that of the amino acids which were followed by histamine and biogenic amine assays.

Microbial Analysis

Butterfield's phosphate-buffer (BPB) was prepared by dissolving 34 g potassium phosphate (monobasic) in 800 mL distilled water. The pH was then adjusted to 7.2 with 1N NaOH, the volume brought up to 1 L with distilled water and then sterilized for 15 min at 121°C. Working buffer was prepared by diluting 1.25 mL of the BPB stock solution, brought to 1 L with distilled water, then sterilized for 15 min at 121°C. The working buffer was used for all serial dilutions during the determination of aerobic plate counts and Niven's (Table 3-2) differential plating.

All microbial analyses were carried out using 20 g of each fish weighed into separate labeled half pint mason jars. To each jar, 180 mL BPB (9:1 v/w) was added and blended for 2 min using a Hamilton Beach Blend Master blender (Hamilton Beach/Proctor-Silex, Inc., Glen Allen, VA) at the "liquify" position. The samples were

Table 3-2. Niven's differential medium composition.

Component	% (w/v)
Bacto-tryptone	0.5
Yeast extract	0.5
L-histidine	2
NaCl	0.5
$CaCO_3$	0.1
Bromocresol purple	0.006
Bacto-agar*	2

*pH of medium was adjusted before addition of agar using 1 N NaOH
Source: Niven et al., 1981.

then decimally diluted with BPB. Four aliquots, each of 0.1 mL of the appropriate serially diluted samples were spread-plated on four separate tryptic soy agar plates, fortified with 1.5% NaCl for aerobic plate count (APC) determination. Pour plates were also prepared for some samples as were deemed necessary. Following incubation of the plates at 37°C for 24 hr, bacterial colonies were counted to estimate the total aerobic plate count population of each fish sample.

Niven's differential medium was also used to confirm the presence of histamine producing bacteria, and to count their numbers. The appropriate serial decimal dilutions prepared, as explained above, were plated on Niven's medium (Niven et al., 1981). Four plates of Niven's differential medium were used for each dilution sampled. These plates were incubated at 37°C for 24-48 hr. Colonies surrounded by deep blue purple halo were identified as histamine producers.

AromaScan Analysis

A 10 g portion of each fish was weighed out and placed in analysis pouches. The pouches were evacuated then charged or inflated with carbon filtered air. The bag humidity setting was 5 g/m^3 and the reference air humidity was at 10 g/m^3. Each bag was individually incubated at 35°C for 10 min so that the head space was equilibrated. Prior to analysis, the sensors were cleaned using reference air. The air was dried by passing it over dried silica gel. After each analysis, the sensors were washed for 1 min with the head space from the wash bottle filled with 2% isopropanol. The sensors were then allowed to react with reference air for 2.5 min before the next analysis. AromaScan

sampling time was 120 sec and data was collected between 45 to 60 sec of the total analysis time. AromaMap patterns (Figure 5-4) were generated from all the samples analyzed.

Total Volatile Base-Nitrogen Analysis

The direct MgO method, as described by Woyewoda et al. (1986), was used for the analysis of total volatile base-nitrogen (TVB-N). The method used steam distillation for extraction of the volatile bases from fish. Depending on the species, a TVB-N value of 30 mg/100 g is considered the threshold of fish spoilage.

Apparatus. Four vertical distillation apparatuses (Kimble Kontes Scientific Glassware/Instrument, Vineland, NJ), illustrated in Figure 3-2, were each heated with a 1L heating mantle (Glas-Col, Terre Haute, IN). Fish samples were homogenized in a West Bend® High Performance household food processor (West Bend processor) (West Bend, WI) and extracted with a Hamilton Beach 14 Blend Master blender (Hamilton blender). Other supplies included two 50 mL buret, two 25 mL pipettes, eight 250 mL and four 125 mL Erlenmeyer flasks, and two 25 mL beakers.

Boric acid solution (2%). Twenty grams of boric acid were dissolved in a 1L volumetric flask using distilled deionized water and then brought up to volume.

Sodium hydroxide (0.1N). Sodium hydroxide (NaOH) solution was prepared by dissolving 4 g NaOH in 800 mL of distilled deionized water and then volumetrically made up to 1L.

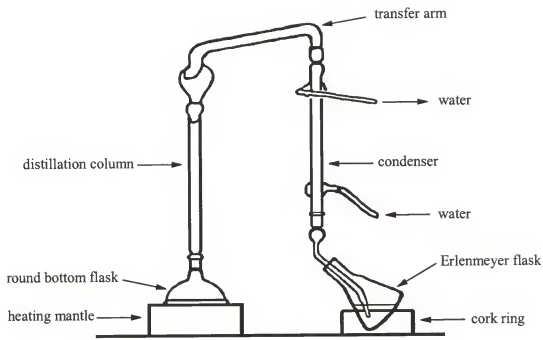


Figure 3-2. Schematic of TVB-N steam distillation apparatus.

Sulphuric acid (0.05N). Sulphuric acid (H_2SO_4) solution was prepared by adding 1.39 mL of concentrated H_2SO_4 to a 1L volumetric flask containing 800 mL distilled deionized water and then brought up to volume with distilled deionized water.

Screened methyl red indicator. To 100 mL ethanol, 0.05 g methyl red and 0.075 g bromocresol green were added, dissolved and mixed thoroughly.

Phenolphthalein indicator. One gram phenolphthalein was dissolved in 100 mL ethanol (95%) using a volumetric flask.

Standardization of 0.1N sodium hydroxide. Potassium hydrogen phthalate (3 g) was put into a 25 mL beaker and dried overnight at 110°C. The beaker was then removed and cooled in a desiccator. The NaOH was standardized by adding 0.30 g potassium hydrogen phthalate into each of three 250 mL Erlenmeyer flasks, to which were added 50 mL distilled water and 3 drops of phenolphtalein indicator. Using a 50 mL buret, the potassium acid phthalate sample was titrated with 0.1N NaOH to the end point of the first visible but permanent pink color, and the normality was calculated as follows:

$$N_1 = (W1 \times 1000)/(204.22^* \times V_1)$$

where: N_1 = normality of NaOH

V_1 = volume (mL) of NaOH used for titration

W_1 = weight (g) of potassium acid phthalate added to each Erlenmeyer flask.

*Molecular weight of potassium acid phthalate

This was repeated for each of the remaining phthalate samples and the average normality of the NaOH was calculated.

Standardization of 0.05N sulphuric acid. Aliquots of 25 mL of the prepared H_2SO_4 were pipetted into each of three 125 mL Erlenmeyer flasks and 3 drops of phenolphthalein were added. Each flask was titrated to the phenolphthalein end point, appearance of the first perceptible but permanent (30 sec) pink color, with standardized 0.1N NaOH. This was repeated for each of the remaining two samples and the volume of

NaOH used was recorded and averaged. The averaged volume was then used to determine the normality of H_2SO_4 using the formula:

$$N_2 = (N_1 \times V_2)/V_3$$

where: N_1 = normality of NaOH

N_2 = normality of H_2SO_4

V_2 = average volume (mL) of NaOH used for titrations

V_3 = volume (mL) of H_2SO_4 added to each Erlenmeyer flask

Extraction of Analyte. The fish sample was homogenized using a West Bend processor. Ten grams of fish homogenate were weighed into each of four half-pint size mason jars. Into each jar, 100 mL of distilled deionized water was added and the sample blended for 2 min using a Hamilton blender. The extract was then transferred to a 1L round bottom flask. The jar and blade were rinsed three times using a total of 200 mL distilled deionized water. All rinsings were added to the flask. Two grams of magnesium oxide and anti bumping granules were added and the flask was swirled. Several drops of antifoam concentrate A were added and the flask connected to the still.

To each of four 250 mL Erlenmeyer receiving flasks, 25 mL of 2% boric acid and three drops of screened methyl red indicator were added. The receiving flasks were installed such that the receiver tubes dipped below the boric acid solution (Figure 3-2). The distilling flasks were heated at a rate such that the liquid boiled in exactly 10 min. Distillation was carried out using the same heating rate for exactly 25 min.

TVB-N Determination. After distillation the solution in the receiver flask was back titrated to the original pink color using the standardized 0.05N H_2SO_4 solution. Four blanks containing all reagents except the sample, which was replaced by 310 mL distilled water, were also prepared and treated as the samples. TVB-N expressed as milligrams nitrogen per 100 g (mg N/100 g) was calculated using the formula:

$$TVB\text{-}N = (V_4 - V_5) \times N_2 \times 100 \times 14)/W_2$$

where: V_4 = volume (mL) of H_2SO_4 used for sample

V_5 = volume (mL) of H_2SO_4 used for blank

N_2 = normality of H_2SO_4

W_2 = weight of sample in grams

*14 is the molecular weight for nitrogen.

Ammonium chloride was used as the standard for fortification in the recovery and reproducibility studies (Wekell et al., 1987), and the results are given in Table 4-3.

Amino Acids

At the preliminary stages of our study the method of Joseph and Marsden (1986) was used, but it was not effective in separating histidine and glutamine in mahi-mahi and tuna. Consequently, it became necessary to develop a method which would effectively allow us to quantify histidine and glutamine in these fish species. During the development of the method we worked with 0.05 M phosphate buffer and the method was used for analysis of free amino acids in several fish species (Antoine et al., 1999). However, in an attempt to further improve the separation of glutamine from the high

levels of histidine in mahi-mahi, acetate buffer was used (Antoine et al., 2000). Hence the study of the changes in amino acid levels was done using 0.05 M acetate buffer as described below .

Mobile phase preparation. Mobile phase A was comprised of 0.05M acetate buffer (pH 5.5) prepared from analytical grade acetic acid, methanol, and tetrahydrofuran (80:19:1). The pH of the acetate buffer was adjusted using 10N NaOH and measured using a Brinkmann Metrohm 632 pH meter (Westbury, NY). Mobile phase B was made of methanol and 0.05M acetate buffer (pH 5.5) (80:20). The mobile phases were filtered using Supor®-200, 47 mm diameter, 0.2 µm filter membrane (Gelman Science, Ann Harbor, MI) and degassed by sparging for 5 min with helium.

OPA-thiol reagent preparation. OPA-thiol reagent was made up at least 24 hr before use by dissolving 27 mg of o-phthaldialdehyde in 500 µL of absolute alcohol and then 5 mL of 0.1M sodium tetraborate (pH 9.5) was added, followed by 50 µL of mercaptoethanol. The mixture was vortexed and stored in a tightly closed container in the dark. The OPA-thiol reagent may be kept for several days with periodic additions of 20 µL of mercaptoethanol to maintain the yield of OPT-amino acid derivatives (Joseph and Marsden, 1986; Miles and Leong, 1992).

Amino acid standards preparation. A stock solution of each standard was prepared by dissolving the equivalent of 2500 nmol in 0.05M NaH_2PO_4 buffer (pH). This stock solution was then used to prepare working solutions from which a calibration curve was prepared.

Sample preparation. Fish samples were homogenized using a West Bend processor. Ten grams of each homogenate were weighed into each of four half-pint size mason jars. To each jar, 40 mL of extracting solvent (75% methanol in distilled deionized water) was added. The sample was blended using a Hamilton blender and transferred to a 100 mL volumetric flask. The jar and blade were rinsed using 3 x 15 mL of the extracting solvent and these were added to the volumetric flask. The flask was brought up to volume and stored for 60 min or overnight at 4°C. The content of the flask was transferred to a centrifuge tube and centrifuged at 15,000 rpm (27,000 x *g*) for 40 min using a refrigerated centrifuge IEC model B-20A (International Equipment Company, Needham Heights, MA). The supernatant was immediately filtered using an Acrodisc CR PTFE syringe membrane filter (0.2 µm, 25 mm) (Gelman Sciences), then diluted and treated as per standard solution.

Instrumentation. The method of Antoine et al. (1999; see chapter 4) was slightly modified (see mobile phase preparation). A Beckman reversed phase HPLC column, Ultrasphere ODS 5 µm particle size, 4.6 mm x 25 cm (Beckman Instruments, Inc., Fullerton, CA), was used without a guard column. Gradient elution was generated using a Bio-Rad (Hercules, CA) solvent delivery system model 2800 equipped with dual pumps, and a Rheodyne injection valve (Model 7125-081) containing a 20 µL sample loop. BioRad ValueChrom Software version 4, 1988-1994, was used for controlling the gradients and flow rate of the two degassed mobile phases. A Perkin-Elmer LC 240 fluorescence detector (Buckinghamshire, England) fitted with a 7 µL flow cell was used with the excitation and emission monochorometers set at 340 nm and 430 nm,

respectively. The other detector settings were response time of 0.7 sec and an attenuation factor of 256. A Spectra-Physics SP 4092 integrator (San Jose, CA) was used to generate chromatograms.

Amino acid derivatization. To 100 µL of amino acid standard or diluted sample extract, 400 µL of OPA was added and mixed thoroughly using a vortex (Figure 3-3). Exactly 2 min after mixing, the sample was manually injected and the gradient run started. It was important that the time between mixing and injecting into the HPLC be consistent (Joseph and Marsden, 1986) because of the limited stability of the OPT-amino acid derivatives. Sodium phosphate buffer (0.05M, pH 5.5) was used for the blank, to dilute all supernatants, and for preparation of standards. The concentration of each amino acid of interest was measured using an external calibration curve. All standards and samples were ran under the same conditions and a room temperature of 21°C. The concentration of each amino acid in the supernatant was calculated based on an external calibration curve. Figure 3-4 illustrates the steps involved in this method for analysis of free amino acids.

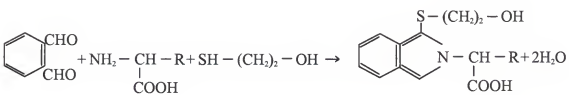


Figure 3-3. Illustration of OPA derivatization reaction with primary free amino acids.

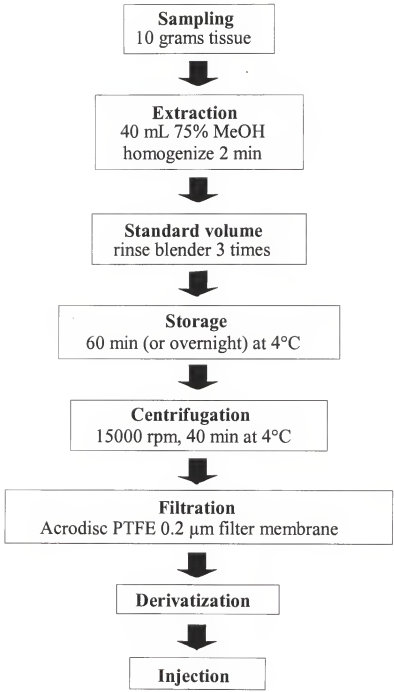


Figure 3-4. Sample preparation flow chart for HPLC analysis of free amino acids.

Calculations. Analyte concentration in the samples was determined using the linear regression equation for the respective amino acid calibration curve as follows:

Conc. in sample (mg/ 100g) = peak height x dilution factor x 100/slope

where slope = coefficient of x in regression equation

Biogenic Amines

The gas chromatographic method of Rogers and Staruszkiewicz (1993) was modified for the analysis of biogenic amines. The modification was based on the work of Luten et al. (1992). It used 75% methanol in distilled water instead of 100% methanol for extraction of the amines. A gas chromatograph temperature gradient program was used for quantification of the biogenic amines, including histamine.

Instrumentation. A PE 8500 gas chromatograph (Perkin-Elmer Corp., Norwalk, CT) was fitted with a DB-1 capillary column, 15 m x 0.32 mm I.D., 3 μm film thickness (J & W Scientific, Folsom, CA). The following temperature gradient program was used: 150°C, 0 min, 5°C/min; 156°C, 3 min, 5°C/min; 161°C, 2 min, 20°C/min; and a final temperature of 300°C held for 5 min. During the developmental stages, it was serendipitously observed that a splitless/split injection made it possible for histamine quantification. Prior to to the use of the splitless/split injection only putrescine and cadaverine consistently eluted. The FID detector temperature was 325°C, and the injection temperature was 300°C. Carrier gas flow rate was 2.45 mL/min with a column pressure of 8 psi. Injection volume was 1 μL and all injections were done manually with a 10 μL gastight syringe (Hamilton Co., Reno, NA).

Calibration curve preparation. Stock solutions were prepared by adding the equivalent of 100 mg pure diamines to separate 100 mL volumetric flasks. The crystals were dissolved in 0.1N HCl and brought up to volume. These standard solutions were stored at -20°C, from which working solutions were prepared fresh weekly. An external calibration curve was prepared using the volumes and concentrations shown in Table 3-3. Standards were derivatized by adding 1 mL of each of the five working standard mixtures to separate 100 mL round bottom flask (RBF). The flask was swirled several times, then evaporated to dryness on a rotary Buchi Rotavapor R114 (Brinkmann Instruments, Inc., Switzerland) at 50°C. The rotavapor was coupled to a KNF Neuberger vacuum pump model 13-878-42 (Trenton, NJ). The residue was washed with 2 mL water and again evaporated to dryness. One milliliter ethyl acetate and 300 µL pentafluoropropionic anhydride (PFPA) were added to the dry residue, stoppered, mixed, and heated in a water bath (Fisher Scientific Versa-bath) at 50°C for 30 min. The mixture was swirled at least once during heating. The PFPA-amine derivative was transferred to 10 mL screw-capped glass tubes and the RBF rinsed with 3 x 1 mL ethyl acetate. The tube was evaporated to dryness under a stream of nitrogen to remove excess reagent, and redissolved in 1 mL 30% ethyl acetate in toluene (EAT).

Sample Extraction Procedures. Ten grams of homogenized fish sample were weighed into a half pint mason jar and 40 mL extracting solvent (75% methanol in distilled water) was added. The sample was blended for 2 min with a Hamilton Beach 14 Blend Master set at the "liquefy" position. The extract was transferred to a 100 mL volumetric flask and the blade and jar were rinsed with 3 x 15 mL of extracting solvent.

Table 3-3. Amine concentrations used in preparation of calibration curves.

	Concentration µg/mL		
Mixture No.	Putrescine	Cadaverine	Histamine
1	5.0	5.0	10.0
2	10.0	20.0	20.0
3	20.0	50.1	40.0
4	40.0	80.2	80.0
5	60.0	100.2	100.0

The rinsings were added to the volumetric flask which was then heated in a water bath at 60°C for 15 min, cooled to 25°C, and brought up to volume with extracting solvent. The extract was centrifuged at 4°C and 27,000 x g (15,000 rpm) for 40 min using an IEC centrifuge. The supernatant was filtered using 0.2 µm Gelman Acrodisc membrane filters (47 mm diameter). All filtrates were put into Falcon polyethylene tubes (Becton Dickinson and Company, Franklin, NJ) and stored at -20°C until analysis.

Sample derivatization. Ten milliliters of extract were pipetted into a 100 mL round bottom flask (RBF), then 0.5 mL of 1N HCl were added and the mixture prepared as per standards prepared for calibration curve.

Analyte separation. The derivatized samples and standards were separated from unwanted residues on a 3 mL alumina-N solid phase extraction (SPE) tubes (Supelco, Bellefonte, PA). The SPE tubes were installed on a rack and conditioned with 2 mL hexane, which was discarded, and immediately loaded with 150 µL of derivatized sample. Eluent collection began when the sample was loaded into the tube. As the sample passed through the frit, 3-4 drops of methanol were added and allowed to pass through the frit. The analyte was eluted with 8 x 2 mL methanol which flowed under

gravity. All effluent was collected, evaporated to dryness, and redissolved in 150 µl of 30% EAT.

Histamine Analysis using the AOAC Fluorometric Method

Preparation of resins. Dowex 1-X8 anion exchange resin was converted to -OH form by adding 15 mL 2N NaOH/g into a 500 mL beaker. The mixture was swirled and allowed to stand for 30 min. The liquid was decanted and the step repeated with additional base. The resin was thoroughly washed twice with distilled deionized water and slurried into fluted filter paper and stored under water. Resin was prepared fresh weekly.

Apparatus. Chromatographic polypropylene tubes 200 x 7 mm (I.D.) (Kontes, Vineland, NJ) were each fitted with 45 cm of Teflon tubing and a flow control valve. The columns were packed with an 8 cm bed of Dowex 1-X8 anion exchange resin 50-100 mesh (Supelco, Bellefonte, PA), and the height of the tubes were adjusted to ensure that the flow rates were the same and > 3 mL/min.

A Sequoia-Turner photofluorometer model 450-005 (Abbott Diagnostics, Abbott Park, IL) was fitted with a 360 nm narrow band pass (NB) excitation wavelength and a NB 440 emission wavelength filters. A 5 mL glass cuvette was used for all measurements.

Extraction Procedures. The same extract as per biogenic amines were used.

Ion Exchange Analyte Separation. Five milliliters of water were passed through the packed column and the eluate discarded. One milliliter of the sample

supernatant was pipetted onto the column followed by 5 mL of water. Column flow was immediately initiated into a 50 mL volumetric flask containing 5.0 mL of 1.0N HCl. When the liquid level was about 2 mm above the resin, 5 mL of distilled water were added and again allowed to elute. This was followed with more water in larger portions until 35 mL were eluted. The column flow was then stopped. The water level was always kept above the bed level. The flask was brought up to volume with distilled water, stoppered and mixed. The eluate was put into 50 mL Falcon polypropylene tubes and stored in the refrigerator.

Determination. Eluate was pipetted into 50 mL Erlenmeyer and proceeded as per standard solutions described below. For samples which contained >15 mg histamine/100 g, an appropriate dilution was done by adding the predetermined volume of the eluate into 100 mL volumetric flasks and brought to volume using 0.1N HCl.

Preparation of solutions. Standard phosphoric acid, 3.64N, was prepared by diluting 121.8 mL H_3PO_4 (85%) to 1 L, according to the AOAC Official Methods of Analysis (15th ed.). Five milliliters of acid were then titrated with standard 1.00N NaOH to the phenolphthalein end point. A 0.1% o-phthaldialdehyde (OPA or OPT) solution was prepared by dissolving 100 mg in 100 mL methanol.

Histamine standard stock solution, 1 mg/mL as free base, was made by weighing 165.1 mg histamine -2HCl into a 100 mL volumetric flask. The contents were dissolved and diluted to volume with 0.1N HCl. The stock solution was stored at -20°C, from which an intermediate solution (10 μg/mL) was prepared fresh weekly. The intermediate solution was made by pipetting 1 mL of stock solution into a 100 mL volumetric flask

and diluted to volume with 0.1N HCl. From this intermediate solution, working standard solutions were prepared daily.

Working solutions of 0.5, 1.0, 1.5 μg/5 mL were prepared by pipetting 1, 2, and 3 mL of the intermediate solution into separate 100 mL volumetric flasks. The flasks were then diluted to volume with 0. 1N HCl.

Calibration and Calculations. Duplicate 5 mL aliquots of each working standard solution (or sample eluate) were pipetted into separate 50 mL Erlenmeyers flasks. To each flask, 10 mL 0.1N HCl was pipetted and mixed. Three milliliters of 1N NaOH was then added and the flask was again mixed. Within 5 min, 1 mL OPT solution was pipetted in and mixed. After exactly 4 min, 3 mL of 3.64N H_3PO_4 was pipetted in and mixed immediately. Eight to ten reactions were run simultaneously by adding reagents to the Erlenmeyers flasks in a set order.

The blank was prepared by substituting 5 mL 0.1N HCl for histamine solution. All fluorescence intensity (I) measurements were done within 1.5 hr of derivatization. A plot of I (corrected for blank) against μg histamine/5 mL aliquot was made, and the slope determined as per protocol, using the formula:

$$\text{slope} = m = [(I_a/1.5) + I_b + 2I_c]/3$$

Histamine concentration was then calculated using the formula:

$$\text{mg Histamine/100 g fish} = (10)(F)(1/m)(I_s)$$

where I_s, I_a, I_b, and I_c = fluorescence from test sample, 1.5, 1.0, 0.5 mg histamine standards, respectively;

F = dilution factor = (mL eluate + mL 0.1N HCl)/mL eluate.

F = 1 for undiluted eluate.

Statistical Analysis

Statistical analysis was carried out using SAS (Software version 6.12, SAS Institute Inc., Cary, NC) mixed procedure for analysis of the changes in the levels of the various factors analyzed (amino acids, biogenic amines, TVB-N, sensory evaluations, and microbial populations), and test of effects. The said system was used for paired difference analysis of the sensory data, and for determination of Pearson correlation coefficients. All standard deviations were determined using Microsoft Excel for Windows 98, graphs were generated using Lotus Freelance Graphics 97 (Microsoft Corp., Seattle, WA) and Sigma Plot for Windows, Version 3.03, 1986-1995 (Jandel Scientific Corp., Chicago, IL). AromaMap patterns were generated by AromScan software (AromaScan, plc., Crewe, UK). This system used the Sammon mapping statistical technique to generate two dimensional patterns in order to visualize gas or odor discrimination.

CHAPTER 4
RESULTS AND DISCUSSION

HPLC Method for Amino Acid Analysis

The lowest detected levels (2 X the noise level) of the amino acid standards of interest were 40 pmol/mL for histidine and lysine, and 70 pmol/mL for ornithine. The inter (day-to-day) assay of the amino acid standards were reproducible with a coefficient of variation of 4.1-8.7%, and intra (within-day) assay of 0.8-3.1%.

A chromatogram of the amino acid standards of interest is shown in Figure 4-1A. Figure 4-1B shows the linearity obtained for the calibration curves of three amino acids. Calibration concentrations ranged from 50-600 pmol/mL for histidine, and 100-800 pmol/mL for lysine and ornithine. Attempts to use an internal standard (norleucine, ϵ-aminocaproic acid, and glycyl-tryptophan) proved frustrating because they coeluted with other peaks.

Three different spike levels were used for the recovery study (Table 4-1). Volumes of standard solutions ranging from 0.5-2 mL were added to 10 g of minced fish, which were then homogenized, extracted, and brought to a standard volume (100 mL). Recovery studies using the mahi-mahi species served as a good model because of its complex matrix of high lipid levels and the widely different levels and large number of free amino acids. All standard solutions and sample dilutions were prepared using 0.05

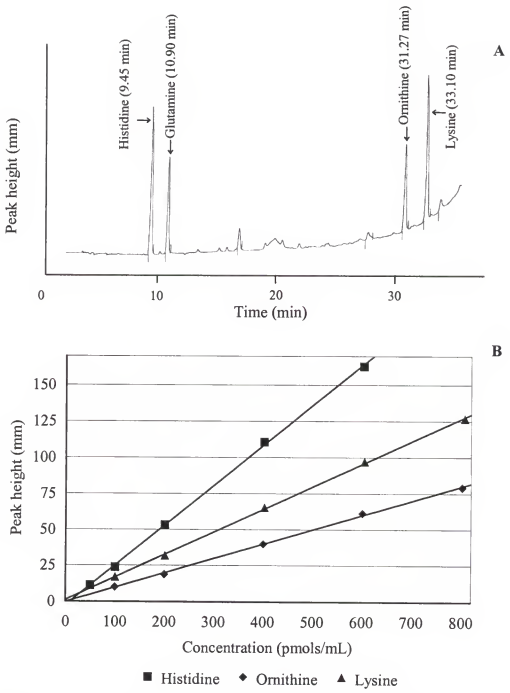


Figure 4-1. (A) Chromatogram of histidine, lysine, and ornithine using phosphate buffer, and (B) their calibration curves. Mobile phase A was 80:19:1 of 50 mM sodium phosphate buffer (pH 5.5), methanol, and tetrahydrofuran. Mobile phase B was composed of 80:20 of methanol and 50 mM sodium phosphate buffer (pH 5.5). Gradient program used was as follows: 0-15% B in 5 min, 15-50% B in 5 min, and 50-100% B in 24 min. Flow rate was 1.5 mL/min, and injection volume was 20 μL.

Table 4-1. Percent recovery of amino acid standards from fish samples.

Amino acid	Spike amount nmol/g (mg/100g)	Recovery[a] (%)
Histidine	62.5 (9.7×10^{-4})	92.8 ± 7.2
	125.0 (1.94×10^{-3})	94.4 ± 8.8
	250.0 (3.88×10^{-3})	92.6 ± 11.3
Ornithine	25.0 (4.22×10^{-4})	95.2 ± 4.8
	50.0 (8.43×10^{-4})	95.7 ± 8.6
	100.0 (1.69×10^{-3})	103.5 ± 2.5
Lysine	25.0 (3.66×10^{-4})	65.6 ± 3.0
	50.0 (7.3×10^{-4})	70.3 ± 2.1
	100.0 (1.46×10^{-3})	82.3 ± 4.7

[a]Mean ± standard deviation from 4 samples taken from each fish, each sample analyzed in duplicate.

M NaH_2PO_4 (pH 5.5). The Association of Biochemical Resources Facility (ABRF) considers that a 90-95% recovery, and 70% in some cases, is expected for OPA pre-column derivatization using RP HPLC, depending on the amount analyzed, the instrument, and the operator (West et al., 1996). The recovery levels obtained for lysine may be due to the instability of the two forms of its OPA derivatives (Simons and Johnson, 1978). Hill et al. (1982) and Simons and Johnson (1977) discussed the possible internal quenching of the fluorescence of each lysine-OPA-mercaptoethanol structure. Turnell and Cooper (1982) reported on the half lives of several OPA-amino acid-mercaptoethanol derivatives. They showed that lysine has a half life of 31.4 min, which is less than its retention time. Umagat et al. (1982) claimed that at pH below 6, detection

of lysine is problematic. They reported improvement in the stability of lysine-OPA-derivative when sodium dodecyl sulfate (SDS) solution (1% v/v) was added to the amino acid samples. However, we did not use SDS in our experiment which adds to the number of steps in the process of a simplified method. We observed that at pH 6.2, lower sensitivity and poor peak symmetry was obtained for the amino acids of interest, particularly lysine. At pH 5.5 and room temperature (21°C), we obtained the best peak symmetry and sensitivity for the analytes of interest in the fish samples. At higher temperatures (30, 35 and 40°C), we observed reduced sensitivity for the amino acids of interest. Cronin et al. (1979) reported similar decreases in fluorescence for amino acids with one hydrogen atom attached to the amino ∝-carbon. Roth (1971) showed that for lysine, fluorescence increases from pH 5-6, is maximum at pH 6-7, then decreases at pH greater than 7, and different amino acids have different pH's at which fluorescence is maximum. In foods, lysine, which contains an ∈-amino group, reacts with reducing sugars, and carbonyl groups produced from lipid oxidation (Belitz and Grosch, 1987; Damodaran, 1996). The N-deoxyketosyl-amino acid derivatives so formed, is the initial step of Maillard reactions which form at room temperature (Kirk, 1979). Due to its reactivity, lysine is lost during processing of foods (Belitz and Grosch, 1987).

Three different fish species were analyzed (Figure 4-2) in order to demonstrate the feasibility of using this method for analysis of fish. The levels of the three amino acids found in the fish samples are shown in Table 4-2. In red muscle fish, mahi-mahi and tuna, significantly higher ($P < 0.05$) levels of histidine were obtained from white tissue than from the red tissue. The histidine in the white tissue was significantly greater ($P <$

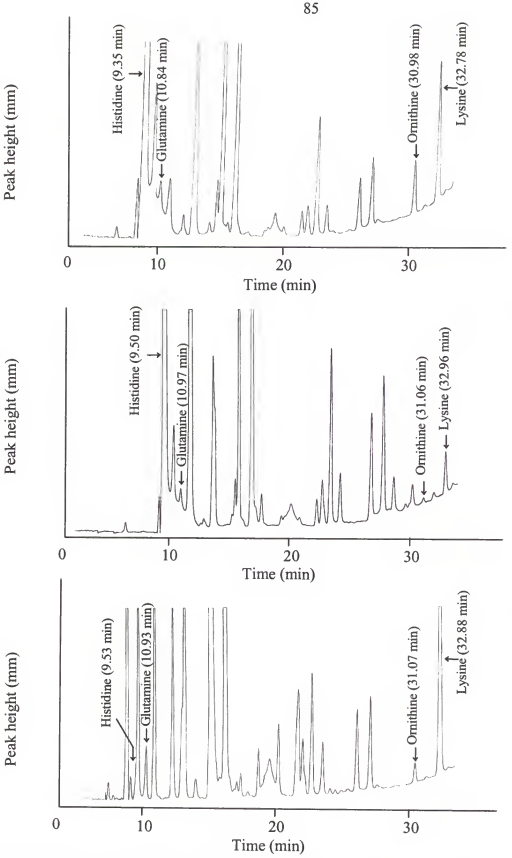


Figure 4-2. Chromatogram of free amino acids in (A) mahi-mahi, (B) bigeye tuna, and (C) flounder samples. Conditions are the same as described in Figure 4-1.

Table 4-2. Free amino acid content[a] (mg/100g) in mahi-mahi (*Coryphaena hippurus*), bigeye tuna (*Parathunnus mebachi*) and flounder.

	Mahi-mahi			Bigeye tuna		Flounder		
	Histidine	Lysine	Ornithine	Histidine	Lysine	Histidine	Lysine	Ornithine
White tissue								
1	541.4 ± 23.9	35.8 ± 1.9	25.9 ± 1.8	595.5 ± 59.3	5.9 ± 0.3	1.0 ± 0.0	23.6 ± 2.1	2.2 ± 0.1
2	414.9 ± 25.4	71.6 ± 3.8	48.7 ± 4.1	708.0 ± 44.1	3.5 ± 0.2	0.9 ± 0.3	30.6 ± 1.5	4.2 ± 1.8
3	251.8 ± 12.7	65.0 ± 6.6	13.9 ± 1.1	554.7 ± 131.2	12.9 ± 2.9	1.3 ± 0.2	15.0 ± 0.3	1.4 ± 0.4
4	498.5 ± 76.7	39.1 ± 2.4	10.8 ± 1.4	481.9 ± 39.9	9.0 ± 0.4	1.5 ± 0.1	9.3 ± 0.6	0.7 ± 0.1
						1.5 ± 0.1	7.4 ± 0.3	0.6 ± 0.1
Red tissue								
1	367.8 ± 25.7	23.9 ± 0.9	16.7 ± 0.7	478.1 ± 42.6	12.0 ± 0.9			
2	213.8 ± 12.0	36.9 ± 7.7	24.9 ± 1.2	490.1 ± 41.0	8.6 ± 0.2			
3	182.9 ± 18.4	28.9 ± 1.4	9.5 ± 2.1	220.9 ± 27.2	12.3 ± 1.1			
4	278.5 ± 14.9	18.1 ± 1.2	8.5 ± 1.6	398.0 ± 35.1	13.5 ± 1.3			

[a]Mean ± standard deviation from 4 samples taken from each fish, each sample analyzed in duplicate.

0.05) in tuna than in mahi-mahi, whereas the difference in the red tissue for both had a value of P = 0.058. Flounder, a white muscle-fish, had very low levels of histidine.

Lysine levels in mahi-mahi were significantly greater ($P < 0.05$) in the white tissue than in the red; but in tuna, the lysine level was higher in the red tissue than the white tissue, although the difference was not significant. Lysine levels were significantly higher ($P < 0.05$) in the white and red tissues of mahi-mahi than in the respective tissues of bigeye tuna. In mahi-mahi, the lysine levels were significantly higher in the white tissue than in flounder. The lysine level in flounder was higher than that in white tissue of bigeye tuna, although not significantly. Lysine levels in flounder, relative to the histidine, were very high.

Ornithine levels in the white tissue of mahi-mahi were not significantly higher than those in the red tissue. In tuna, ornithine levels were too low to be measured without excessively loading the column. In flounder, ornithine levels were significantly lower than those in the white tissue of mahi-mahi.

Hibiki and Simidu (1959) obtained 1010 mg/100g of histidine in bigeye tuna. It was not clear if they measured combined or free histidine in this red muscle fish. Lukton and Olcott (1958) using ninhydrin as well as the Pauly diazotized sulfanilic acid reaction obtained 481.1 mg/100g of free histidine in white tissue of bigeye tuna. They used 1% picric acid as the extracting solution but also noted that the use of an 80% methanol extracting solution gave similar quantitative results. The above values demonstrated that considerable variations in amino acid levels can be obtained from the same fish species. Lukton and Olcott (1958) concluded that large variations in the imidazole content,

including histidine, were observed with different fish of the same species, but consistently observed that white tissue contained more imidazole compounds than red tissue. Our data also showed large variations from fish to fish of the same species. Such variations are possibly a result of several factors including differences in feeding, season, sex, and stage of maturity (Sakaguchi and Kawai, 1970a; Fletcher et al.1995). Overall, the data gives an idea of the levels of the free amino acids that can be found in the species.

Yoshinaga and Frank (1982) reported that histidine is uniformly distributed in fresh skipjack tuna (*Katsuwonus pelamis*) at concentrations of 564-611 mg/100g. However, Abe (1983) obtained free L-histidine levels of 1389 mg/100g in white tissue of skipjack tuna, and 268.5 mg/100g in red tissue. They used 1% picric acid extraction solution and found that the overall L-histidine content in white tissue of red muscle fish ranged from 15.8 to 92.8 nmol/g. Our data showed higher levels of histidine in the white tissue of tuna. Mukundan et al. (1979), using the standard microbiological assay, found that the levels of L-histidine and lysine in the white tissue of tuna (*Euthynnus affinis*) were twice as great as that of red tissue. We found that histidine and lysine levels in the white tissue of mahi-mahi were consistently greater than those in red tissue, but this was not so for tuna. Ito (1957) used the microbiological assay and an aqueous alcoholic extract and found 563 and 296 mg/100g of histidine in white and red tissues, respectively, for mackerel (*Scomber japonicus*). However, similar levels of lysine (22 mg/100g) were found in both tissue types. Konosu et al. (1974) used TCA extraction solution, and obtained 289 and 54 mg/100g of free histidine and lysine, respectively, from jack mackerel muscle extracts. However, the common mackerel had 676 and 93 mg/100g of

histidine and lysine, respectively, and 5 mg/100g of ornithine. They found 1, 3, and 17 mg/100g of histidine, ornithine and lysine, respectively, in flounder. These latter values are close to our flounder data.

Sakaguchi et al. (1982), looking at free amino acids in yellowtail tuna (*Seriola quinqueradiata*), obtained more than 1000 mg/100g of histidine and about 21 mg/100g of lysine in white tissues. They claimed red tissue had 28 mg/100g of histidine, which was far less than previously reported for some of the red muscle fish (200-400 mg/100g). Their reported lysine level was less than 5 mg/100g in red tissue.

Takagi et al. (1969) demonstrated that histamine formation was greater in white tissue where histidine levels were higher than red tissue. They concluded that the degree of histamine formation tends to be governed by the histidine content, but is not proportional to the loss of histidine. Fletcher et al. (1995) also found large variations in histidine and histamine levels in fish caught simultaneously at the same place and subjected to the same storage regime.

The level of histidine is very low in flounder because it is not a red muscle fish. However, relative to histidine, the level of lysine is very high. Konosu et al. (1974) found 1 mg/100g of histidine, 17 mg/100g lysine, and 3 mg/100g ornithine in flounder, which are similar to our data.

Results of the study of mahi-mahi stored at 7°C.

Microbial. Figure 4-3 shows the microbial growth observed for the uninoculated and inoculated fish samples during storage 7°C. There was no significant difference (P = 0.05) in the microbial levels between the two groups of fish. As storage time increased, there was an expected significant (P = 0.05) increase in the microbial number. On day 3, the samples reached 10^6 CFU/g, which is the point most fish are considered spoiled because of the heavy microbial load (Liston, 1982). This illustrates why fresh fish should not be kept in the refrigerator for greater than 2-3 days before cooking (Woyewoda, 1990).

We were able to determine the histamine producers only for the inoculated fish samples, using the Nivens's differential medium. This was because bacterial colonies isolated in the uninoculated samples were not observed to have the purple halo after 24 hr of incubation, as did the inoculated group. When the uninoculated plates were reexamined 36-48 hr later, individual colonies with purple halos were not discernable because the entire plates were a virtual solid purple color, indicating that the purple halos merged into each other. These observations also seemed to indicate that the histamine producers in the uninoculated samples were of a different species from the *Morganella morganii*, or possibly a combination of slower growing histamine producing species, and/or may reflect the competitive effect of other microflora on the histamine producers in the uninoculated samples. No attempt was made to identify the bacteria in the uninoculated mahi-mahi. Eitenmiller et al. (1981) and Ienistea (1973) explained that histidine decarboxylase, and possibly other amino acid decarboxylases, from different

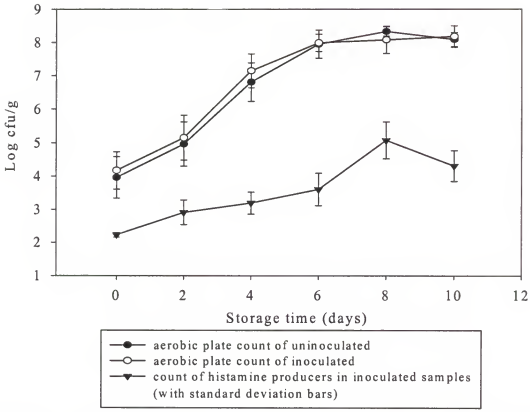
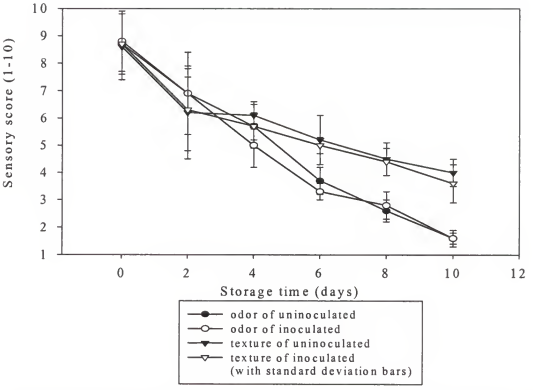


Figure 4-3. Growth of aerobic and histamine producing bacteria in mahi-mahi during storage at 7°C.

organisms are not of equal activity, thus the difference in time for the appearance of the halo in the inoculated and uninoculated fish samples.

Sensory Evaluation. A category scale of 1-10 was used for sensory evaluation where a score of 1-2 indicated very poor and 9-10 was excellent quality. On day 0 fish samples were in very good condition but as storage time increased, the sensory scores decreased (Figures 4-4 and 4-5). The pattern of sensory changes was similar for both the



Figuire 4-4. Sensory odor and texture scores of mahi-mahi during storage at 7°C.

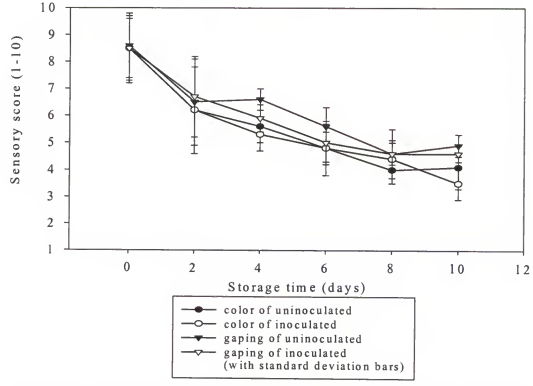


Figure 4-5. Sensory color and gaping scores of mahi-mahi during storage at 7°C.

uninoculated and inoculated groups, and there was no significant difference ($P = 0.05$) between the scores for the uninoculated and inoculated samples for each attribute. The decrease in the scores for the attributes (odor, texture, color, and gaping) was significant ($P = 0.05$) over storage time.

In the case of sensory odor scores (Figure 4-4), the decrease in quality is reflective of the malodorous compounds generated during chilled fish storage (Hultin, 1984). The decline in sensory scores was faster than for the other attributes. In the case of texture and gaping (Figure 4-5), the quality decrease reflects the decrease in firmness (Pigott and Tucker, 1990), and increase in the level of myotomic separation or breakdown of connective tissues of the muscle (Hultin, 1984; Buttkus and Tomlinson, 1966). By day 3, the attributes were given an average score of 6, which corresponds to the score Hansen (1980) describes as the score (on a scale of 1-10) given to the quality of regularly eaten fish.

AromaScan. The AromaMap (Figure 4-6) shows the general shift in the aroma profile for mahi-mahi as storage time increased. The pattern is similar for both uninoculated and inoculated groups of fish, and shifts away from the point of reference (day 0 fish). The map thus reflects the change in fish aroma profile with the passage of time. A similar pattern was shown by AromaScan plc. (Hollis, NH) in which a distinct shift in the aroma profile was observed for fresh and spoiled mahi-mahi.

Total Volatile Base-Nitrogen. TVB-N recovery from fortified mahi-mahi samples was studied using NH_4Cl as a standard (Wekell et al. 1987). The recovery efficiencies ranged from 92 to 108% (Table 4-3). The intra assay coefficient of variation

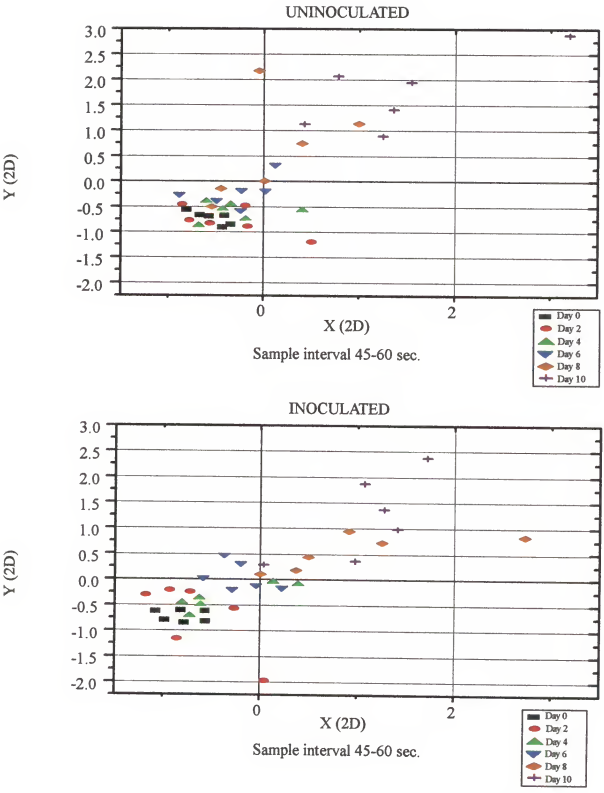


Figure 4-6. AromaMaps of uninoculated and inoculated mahi-mahi stored at 7°C for 0, 2, 4, 6, 8 and 10 days.

Table 4-3. Recovery efficiency of TVB-N standard from mahi-mahi using the steam distillation method.

Fortification level	Fortification amount (mg) N*	Recovery (%)	Mean (%)	Std. dev. (%)	C.V. (%)
Low	5	108.1	102.8	6.9	6.7
		92.7			
		105.3			
		105.1			
Medium	20	92	96.9	4.8	4.9
		93.6			
		101.4			
		100.5			
High	40	99.8	100.0	2.1	2.1
		97			
		101.6			
		101.4			

*NH_4Cl was used for fortification (Wekell et al., 1987).

ranged from 1.2 to 3.5%, and the inter assay from 1.4 to 7.7%. Linearity of the pure standard was assessed over the range from 5 mg/100g to 50 mg/100g and had a $r^2 = 0.99$.

The pattern of TVB-N development was similar for both the inoculated and uninoculated mahi-mahi samples, and there was no significant difference ($P < 0.05$) between the two groups. During the first two days of storage, no considerable change occurred in TVB-N levels (Figure 4-7). On day 3, the level of TVB-N reached 30 mg/100g, a value considered to be the threshold of spoilage (Farn and Sims, 1986). This value corresponds to the microbial level of 10^6 CFU/g observed in Figure 4-3, and the

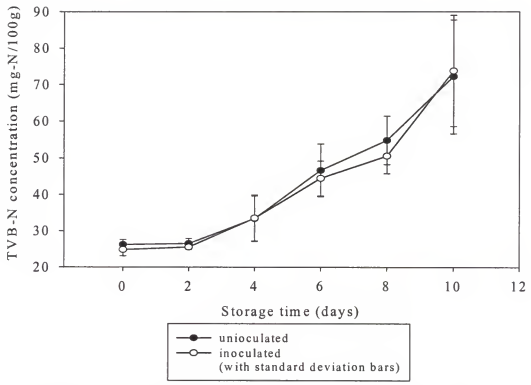


Figure 4-7. Changes in TVB-N levels during mahi-mahi storage at 7°C.

sensory odor score of 6 (Figure 4-4). Liston (1982) pointed out that it is primarily bacterial activity which leads to the characteristic symptoms of fish spoilage. However, during the following few days of storage, the level quickly increased. This observation also illustrates the need to use refrigerated fish within 2-3 days.

Liston (1982) stated that oxidative deamination, the primary mode of NPN utilization, seemed to result in an accumulation of ammonia. The TVB-N results observed agreed with the reports of Wheaton and Lawson (1985), Hultin (1984), and Liston (1973 and 1982) who stated that as the changes intensify, ammonia-like and other off odors are generated. Barker (1981) reported that ammonia is one of the primary

products of bacterial breakdown from ornithine, glutamine, and lysine, but Shewan et al. (1971) clinched the idea when they said that formation of volatile bases is one of the most characteristic features of fish spoilage.

Free amino acids. Minimum detection levels of standard solutions of free histidine, glutamine, ornithine and lysine were 81.0, 35.1, 30.1 and 40.6 pmol/mL respectively. The intra and inter assays had a C.V. of 1.2 to 6.5% and 1.0 to 11.5% respectively. Figure 4-8 shows a calibration curve ($r^2 = 0.99$) of the amino acid standards using acetate buffer in the mobile phase. The recovery efficiencies (Table 4-4) of the standards from fortified mahi-mahi samples ranged from 75 to 99%.

There was no significant difference ($P = 0.05$) between the amino acid levels for the uninoculated an inoculated groups. The change in levels of the amino acids over time was significant ($P = 0.05$) (Figures 4-9 to 4-11). The decline in lysine was faster than that of free ornithine (Figure 4-9), and probably is a reflection of the rate of decarboxylation of this amino acid, and therefore, the rate of formation of its corresponding amine. Ababouch et al. (1991) also reported a similar decrease in lysine levels in sardines during storage at 8°C. As seen later (Figure 4-17), there is good correlation between lysine loss and cadaverine formation, but not between ornithine and putrescine.

In both the uninoculated and inoculated groups, glutamine (Figure 4-10) like ornithine (Figure 4-9), was of relatively low concentration. However, despite their low levels the decrease in the levels of these two amino acids over the period of storage were significant ($P = 0.05$).

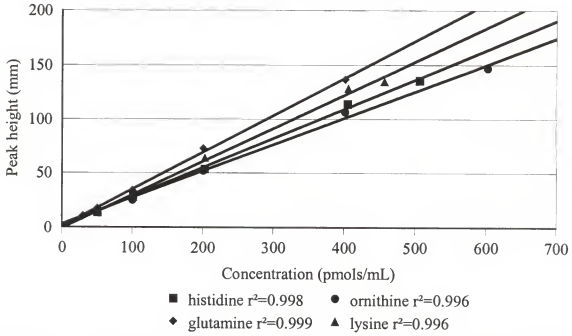


Figure 4-8. Calibration curves for histidine, lysine, ornithine and glutamine using acetate buffer. Mobile phase A was 80:19:1 of 50 mM acetate buffer (pH 5.5), methanol, and tetrahydrofuran. Mobile phase B was 80:20 of methanol and 50 mM acetate buffer (pH 5.5). Gradient program used was: 0-5% B in 5 min, 5-8% B in 5 min, 8-40% B in 10 min, and 40-100% B in 24 min. Flow rate was 1.5 mL/min, and injection volume was 20 µL.

Table 4-4. Recovery efficiency of amino acid standards from mahi-mahi using HPLC OPA precolumn derivatization.

Amino acid	Fortification amount (nmol/g)	Recovery (%)	Std. dev. (%)	C.V. (%)
Histidine	62.5	99	11.4	11.5
	125	85.4	6.9	8.1
	250	90.4	16.3	18.0
Glutamine	25	87.4	3.8	4.3
	50	83.1	3.3	4.0
	100	84.9	8.7	10.2
Ornithine	25	89.6	10.8	12.1
	50	75.8	3.2	4.2
	100	75.6	8	10.6
Lysine	25	97.9	14.5	14.8
	50	78.7	8	10.2
	100	77.8	11.4	14.7

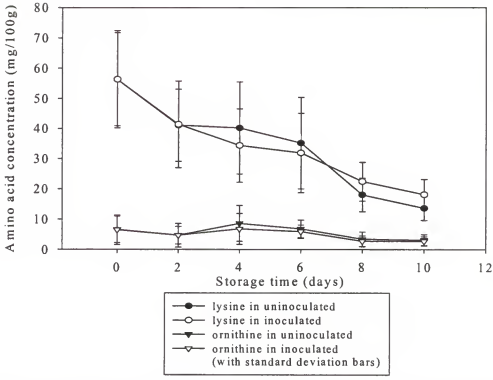


Figure 4-9. Changes in free lysine and ornithine levels during mahi-mahi storage at 7°C.

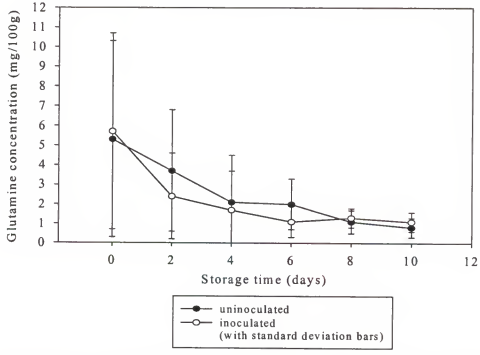


Figure 4-10. Changes in free glutamine levels during mahi-mahi storage at 7°C.

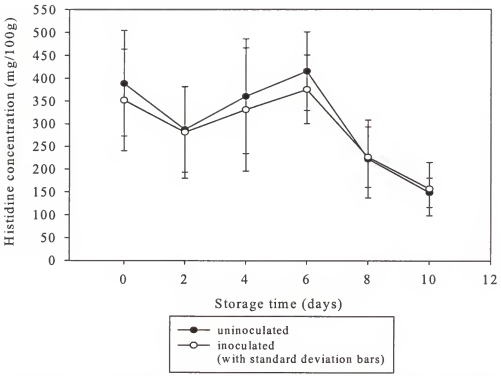


Figure 4-11. Changes in free histidine levels during mahi-mahi storage at 7°C.

The histidine levels (Figure 4-11) were greater than those of lysine, and the levels significantly ($P = 0.05$) decreased over the storage time. The decrease in histidine levels is reflective of histamine formation and other histidine derivatives (Figure 2-5), which result from microbial and enzymatic activities. Ababouch et al. (1991) and Mendes et al. (1998) observed similar decrease in histidine during ice storage and ripening of sardines.

The overall decrease in free amino acids agrees with Liston (1982), who reported a sharp decrease in most amino acids between 4 and 7 days of storage on ice. This decrease corresponded to the period of rapid increase in bacterial count, and is similar to our observations (Figures 4-9 to 4-11). Ababouch et al. (1991) reported a similar decrease in basic amino acids.

Histamine. The AOAC fluorometric method for histamine analysis had intra and inter assays of 0.1 to 2.2% and 3.2 to 6.7% respectively. The mean recovery efficiency (Table 4-5) was 90%.

Table 4-5. Recovery efficiency of histamine standard from mahi-mahi using the AOAC fluorometric method.

Fortification level	Fortification amount mg/100g	Conc. measured mg/100g	Recovery (%)	Std. dev. (%)	C.V. (%)
Low	5	4.6	90.6	5.8	6.4
Medium	10	8.9	89.1	2.9	3.3
High	50	45.4	90.6	3.3	3.6
Mean			90.1		

The graphs of Figure 4-12 show the pattern of histamine development in mahi-mahi stored at 7°C, and the pattern is similar for both the inoculated and uninoculated samples. The difference between the levels for the uninoculated and inoculated samples is significant ($P = 0.05$), even though there was no significant difference between the histidine levels for the said samples. This difference in the histamine levels is probably accounted for by the difference in the enzymatic activities of the micoflora in the uninoculated and the inoculated samples. Behling and Taylor (1981) reported that *K. Pneumoniae* has a higher potential for histamine production at 7°C *than P. morganii*.

Already on day 3, histamine levels reached 5 mg/100g (50 ppm), and increased rapidly after day 4. This is the level of histamine the FDA considers the defect action level (DAL), which implies that some degree of decomposition has occurred in the fish. For the first two days of storage at 7°C, histamine levels remained at or below the level of

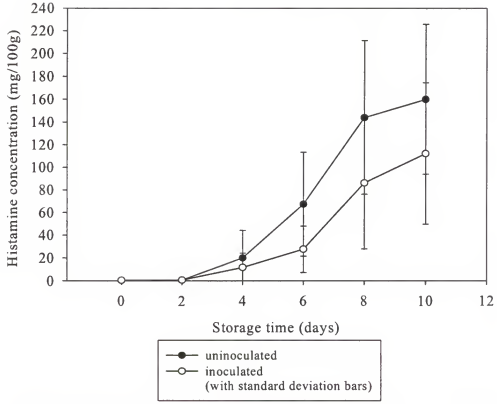


Figure 4-12. Changes in histamine levels during mahi-mahi storage at 7°C.

1 mg/100g (10 ppm), which is normally expected in wholesome fish (FDA, 1995a). The histamine level reached 5 mg/100g on day 3, which corresponds with the TVB-N level of 30 mg/100g (Figure 4-7), the microbial level of 10^6 CFU/g (Figure 4-3), and the cadaverine level of 3 mg/100g (Figure 4-15).

High variation in the histamine levels was observed within the same fish species (Figure 4-12). This is because histamine is known to be unevenly distributed in the fish muscle tissue (Lerke et al., 1978, Etkind et al., 1987). Considerable variation in the levels of histamine is also reported in the literature (Rogers and Staruszkiewicz, 1997; Sims et al., 1992; Walters, 1984). As such, any detection of histamine levels greater than the DAL confirm the presence of decomposed fish tissue (Rogers and Staruszkiewicz,

1997; FDA, 1995a), but histamine is not a reliable indicator of the degree of fish quality (FDA, 1995a; Sims et al., 1992; Walters, 1984). Takagi et al. (1971) found that the rate and level of histamine formation varied with fish species, which makes it necessary to stipulate different DALs for different species (FDA, 1995a). Unlike cadaverine and putrescine, which tend to always be present in spoiled fish (Rodriguez-Jerez et al., 1994; Middlebrooks et al., 1988), histamine is not always present.

Yamanaka (1990) stated that a high level of histidine decarboxylase activity leads to a high level of histamine, and it is known that enzyme activity is influenced by the substrate concentration (Whitaker, 1994; Robyt and White, 1990). Histidine must be in excess of the normal growth requirement, and pH values of 5 to 6 are necessary for maximal induction of histidine decarboxylase (Morris and Fillingame, 1974). In addition, temperature and time abuse influence the rate of histamine formation (Behling and Taylor, 1982). It is, therefore, understandable that in view of the factors that affect histamine production, and the wide variations in the concentrations of free histidine in red muscle fish, histamine levels can be expected to vary widely and, thus, not be a reliable biochemical indicator of fish quality. FDA requires that samples with histamine levels of 2 to 5 mg/100g must be considered suspect and further tests must be conducted for decomposition (FDA, 1995a).

Comparison of Histamine Measured by GC-FID and AOAC Fluorometric Method

Figure 4-13 shows a comparison of the histamine levels as measured by the AOAC and the GC-FID methods. The GC values were not significantly lower ($P > 0.05$)

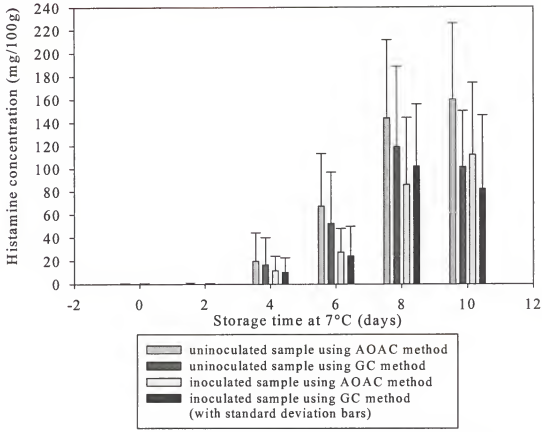


Figure 4-13. Comparison of histamine values in mahi-mahi using AOAC fluorometric and GC methods.

than those of the AOAC and is reflective of the lower recovery efficiency of the GC method for histamine. The GC-FID intra and inter assays coefficient of variation values for histamine were 2.1 and 9.3%, respectively. The minimum standards detected for histamine was 7.0 µg/mL, and the mean recovery efficiency of 67% (Table 4-6). A temperature gradient program was used for separation (Figure 4-14) of the analytes.

Staruszkiewicz and Bond (1981) reported that histamine did not chromatograph easily by GC. They used an oven temperature of 170-180°C for amine separation. Several authors have studied biogenic amines using the GC and either did not investigate,

Table 4-6. Recovery efficiency of biogenic amine standards from mahi-mahi using GC-FID.

Biogenic amine	Fortification amount (mg/g)	Recovery (%)	Std. dev. (%)	C.V. (%)
Histamine	20	66.8	7.6	11.4
	50	77	6.4	8.3
	100	58.1	8.6	14.8
Cadaverine	20	108.3	6.1	5.6
	50	99.1	3.6	3.6
	100	103.6	2.9	2.8
Putrescine	10	100.6	11.5	11.4
	20	110.7	15.5	14.0
	50	96.3	4.4	4.6

or used an alternative method, for histamine (Fardiaz and Markakis, 1979; Staruszkiewicz and Bond, 1981; Rogers and Staruszkiewicz, 1997). These reports testify to the fact that histamine is not easily chromatographed. Mita et al. (1979) reported that because of chromatographic tailing, histamine quantification using the GC can be difficult.

On the other hand, Henion et al. (1981) reported that the trimethysilyl derivative of histamine in tuna was readily resolved using a capillary column. Navert (1975) and Mahy and Gelpi (1977) reported methods which adequately separate histamine and other imidazole derivatives using GC-FID. Doshi and Edwards (1979) reported excellent gas chromatographic properties of histamine and methyl-histidine, and Wada et al. (1982) reported recovery efficiencies of 104-113% for histamine.

Using a gradient temperature program for elution of biogenic amines, we found that histamine chromatographed just as putrescine and cadaverine, but required a

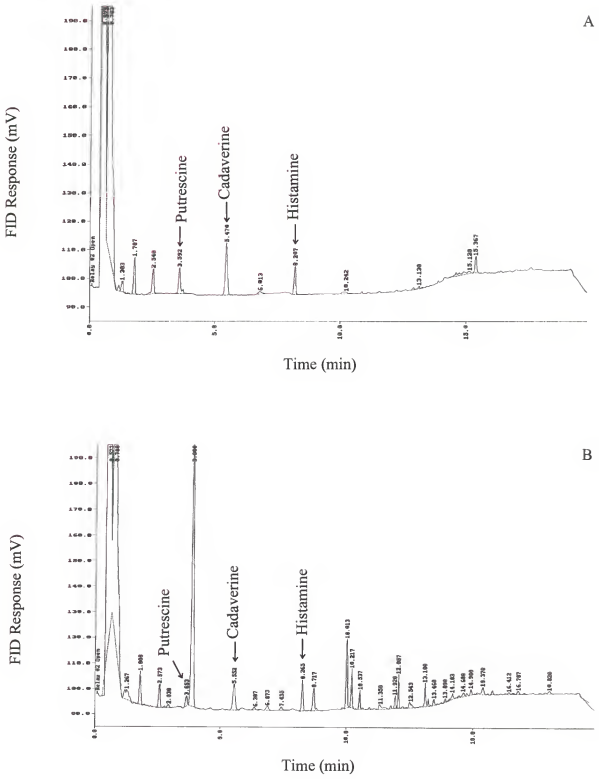


Figure 4-14. Gas chromatograph of (A) biogenic amine standards, and (B) biogenic amines in mahi-mahi stored at 7°C.

splitless/split injection. The results of the GC method for histamine are comparable with the reports of several authors. Mita et al. (1980), using the GC-MS, reported a 70% recovery rate and concluded that his procedure would be applicable for the determination of histamine. Comparable efficiencies were reported by Salazar et al. (2000). Davis et al. (1978), using an HPLC method had a recovery efficiency of 54.4% for histamine. Hungerford et al. (1990) reported much higher sensitivity for measurements of histamine using a flow injection analysis method than using the AOAC method. They reported that extraction efficiencies were influenced by sample matrices.

Fales and Pisano (1962) investigated various factors that influence the successful separation of biological amines (including histamine) using GC. They reported that the injector temperature, solvent type, film thickness of the column, and combinations of air, light, and heat were factors that affect recovery efficiencies. They demonstrated that thermal degradation during chromatography, however, was not a problem. Hiemke et al. (1978) reported that because the derivatized histamine is stable only in the absence of water, up to 50% of the derivatives can, therefore, be lost during preparation.

Cadaverine and putrescine. The GC-FID intra and inter assays coefficient of variation values were 2.4 and 4.6% for cadaverine respectively, and 6.2 and 9.3% for putrescine. The minimum standards detected for these amines were 1.0 μg/mL for cadaverine and 0.5 μg/mL for putrescine. The mean recovery efficiencies for these biogenic amine standards (Table 4-6) ranged from 96 to 110%. Figure 4-14 shows a typical chromatograph of biogenic amine standards and mahi-mahi samples.

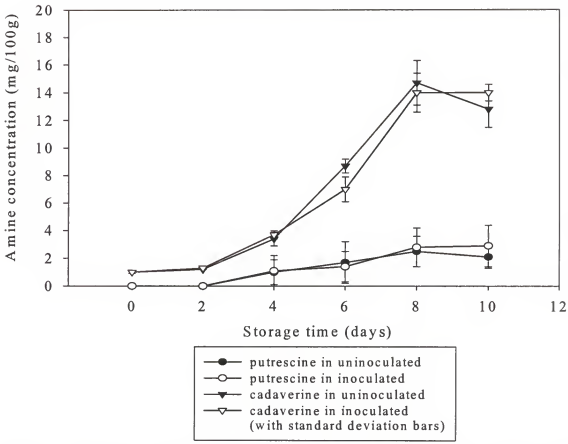


Figure 4-15. Changes in cadaverine and putrescine levels during mahi-mahi storage at 7°C.

The graphs for cadaverine and putrescine levels (Figure 4-15) during mahi-mahi storage at 7°C show that cadaverine formation begins before putrescine and histamine, and that cadaverine levels increased steadily over this storage period. The histamine increase began late and was rapid. Putrescine increase also began late but its rate and level were lower than for cadaverine and histamine. There was no significant difference ($P = 0.05$) between inoculated and uninoculated for levels of cadaverine and putrescine.

Yamanaka (1990) and Ritchie and MacKie (1980) observed a similar pattern of increase for histamine, cadaverine, and putrescine. Ritchie and MacKie (1980) also observed that cadaverine and histamine were two of the major amines formed, and that

histamine was formed later than cadaverine and putrescine. They, too, reported that histamine concentration reached a higher level than cadaverine and putrescine. Baranowski et al. (1990) also found that the pattern of biogenic amine formation is similar at different temperatures, although the levels and rates of formation are slower at lower temperatures. Yamanaka (1990) concluded that during decomposition, levels of each biogenic amine increase at different rates.

Morris and Fillingame (1974) explained that decarboxylases for lysine are adaptive, and that acidic pH and the presence of substrate favored their induction. They also stated that in the absence of ornithine decarboxylase, cadaverine formation is greater. The results show that cadaverine formation began early and increased steadily during the period of storage.

Cadaverine forms in a wide range of fish and shellfish species including tuna, mahi-mahi, cod, octopus, shrimp, squid, etc (Rogers and Staruszkiewicz, 1997; Yamanaka, 1990). Given that cadaverine correlates well with TVB-N, histamine, and sensory odor and Aerobic plate count (Table 4-7) it is suggested that cadaverine by itself, be considered a good indicator of incipient and late spoilage of mahi-mahi. It may also be possible that cadaverine can be used as an indicator incipient and late spoilage for other fish species, but further research will be needed..

Putrescine, like cadaverine, is produced by many different bacterial species (Table 2-3). The graphs of Figure 4-15 show that putrescine increase begins after, and is at lower concentration, than cadaverine. Putrescine is produced by either of two decarboxylases, an induced and noninduced (Tabor and Tabor, 1984) which are adaptive,

require an acidic pH, and a high enough concentration of ornithine. Because of its late formation, like TVB-N, putrescine may also be a good indicator of late spoilage. Rogers and Staruszkiewicz (1997) pointed out that both cadaverine and putrescine are good indicators of tuna and mahi-mahi quality. Putrescine in mahi-mahi, however, may not be a good indicator of incipient spoilage because of its late formation and relatively low levels.

Some Observed Correlations

The following section discusses correlations observed between some of the analytical factors. The correlations examined were those deemed to be of importance because of the relationship between the pairs of data sets examined (Table 4-7).

A correlation describes the measure of the relationship between two variables neither of which may be looked at as a consequence of the other (Snedecor and Cochran, 1961). It is generally designated by the symbol r, whereas regression is designated by the symbol r^2. Correlation differs from regression in that regression refers to the dependence of one variable (e.g. Y) upon the other (e.g. X) such that it is possible to estimate the value of the one (Y) from a knowledge of the other (X). As such, correlation makes no distinction between explanatory and response variables, unlike regression (Moore and McCabe, 1998).

Correlation seeks to determine if two sets of data move together, and is a measure of the strength of interdependence or relationship of the two sets of data (Ott, 1992; Moore and McCabe, 1998). As such, increasing values of one set may be associated with

Table 4-7. Table of correlation (r) values.

Attributes	Cadaverine	Putrescine	Histamine	TVB-N	Lysine	APC
Histamine	0.78	0.72		0.74	0.63	
Putrescine	0.85					
Histidine			-0.43			
Lysine	-0.62			-0.69		
Ornithine		-0.27				
TVB-N	0.74	0.80				
APC	0.70	0.71	0.60	0.71	-0.74	
Odor	-0.78	-0.75		0.84		-0.93

increasing values of the other, resulting in a positive correlation. If decreasing values of one set are associated with increasing values of the other, a negative correlation exists. Correlation may therefore range from -1 to +1. When the relationship or values of both sets of data are unrelated, the correlation nears zero. A correlation is usually proportional or linear, and each field of research has its own range of coefficients that are considered a high level of correlation. Hollingworth and Throm (1982) considered a r value of 0.71 a highly significant correlation. Snedecor and Cochran (1961) cautioned, however, that it is well not to speculate about cause and effect unless (r) $R^2 = 0.8$. There is no simple solution, however, to the cases where a strong but non-linear relationship exists.

A correlation is generally first identified by observing the scatter plot, but in nonlinear cases, the variables are not connected by a simple relation. A strong non-linear relation between two sets of data may be indicative of the influence of two or more variables on a third factor (Snedecor and Cochran, 1961). Strong non-linear relation, therefore, often may be indicative of complex relationships. One thus tries to identify the

specific function that best describes the curve. The fitting of curves to nonlinear data may be for one or more of several reasons. Snedecor and Cochran (1961) point out that the reasons may be the desire to make a good estimate of the dependence of one variable on another; to test or discover a law that relates the variables; or merely the elimination of inaccuracies which nonlinearity of regression may introduce into a correlation. Graphical devices and statistical programs are often used to determine the function that best describes the non-linear relationship observed between pairs of data sets. Sometimes the relation may be rectified by applications of other statistical methods (Snedecor and Cochran, 1961).

Histamine correlation with histidine. The following correlations were obtained by combining all corresponding uninoculated and inoculated data points. Figure 4-16 shows that the relationship between histamine and histidine is not proportional ($r = -0.43$, $P < 0.001$). This implies that as the histidine level decreases, histamine level increases, but not proportionally, and concurs with the results of Fletcher et al. (1995), Clifford et al. (1989), Taylor et al. (1984), and Takagi et al. (1971). This weak correlation reflects that other factors influence histamine development and that histidine is involved in other metabolic pathways (Figure 2-5). For example, histidine is utilized for the production of methylhistidine, imidazolepyruvic acid, urocanic acid, and other imidazole derivatives (White et al., 1964). It has also been established that histamine formation is dependent on the activity of the enzyme involved (Tabor and Tabor, 1984), on temperature and time abuse, and on the level of free histidine in the medium (Yamanaka, 1990; Eitenmiller and De Souza, 1984). Histidine decarboxylase is known to be present in a few microbial

species (Table 2-2). Based on the forementioned factors, it is understandable why our results show that histamine formation is not proportional to the loss of histidine.

Cadaverine correlation with lysine. The correlation between cadaverine and lysine is linear ($r = -0.61$, $P < 0.001$) (Figure 4-17), and indicates the degree of dependence of cadaverine on lysine. Many bacteria are capable of producing the lysine decarboxylase enzyme (Table 2-3), and all fish tissue contains lysine. In addition, as observed in our experiment, cadaverine formation begins early and increases steadily during storage. These factors make cadaverine a good indicator of fish quality both at the incipient and late stages of spoilage, and supports the recommendations of Rogers and Staruszkiewicz (1997) that cadaverine be considered an indicator of mahi-mahi quality.

Putrescine with ornithine. Even though putrescine formation is dependent on ornithine, a poor correlation ($r = -0.27$, $P < 0.05$) exists between ornithine and putrescine (Figure 4-18). This is understandable as much as ornithine is also metabolized into spermine and spermidine (White et al., 1964; Lehninger et al., 1993). Putrescine by itself, however, may not be a good fish quality indicator if only limited changes occur in its concentration (Slemr and Beyermann, 1984). Ababouch et al. (1991) reported insignificant levels of putrescine in sardines stored at 8°C. However, putrescine can be a useful biochemical quality parameter if used in conjunction with cadaverine (Mietz and Karmas, 1978), or included in a summation index of biogenic amines (Slemr and Beyermann, 1984; Mietz and Karmas, 1978).

Histamine with cadaverine and putrescine. The increase in histamine concentration was observed to correlate with the increase of cadaverine ($r = 0.78$, $P <$

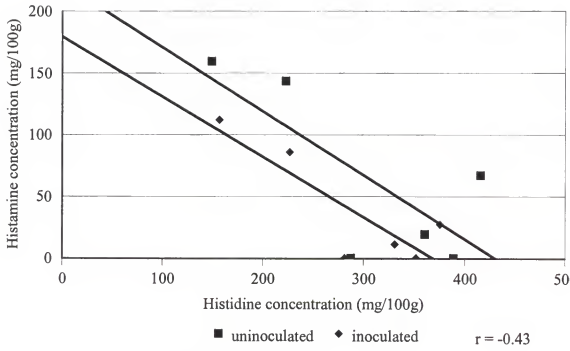


Figure 4-16. Histamine correlation with histidine.

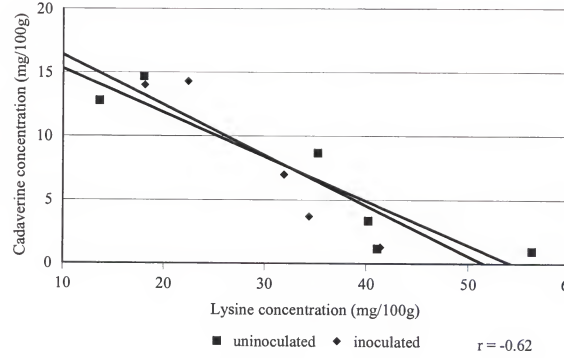


Figure 4-17. Cadaverine correlation with lysine.

0.001) and putrescine ($r = 0.72$, $P < 0.001$) (Figure 4-19). Good correlation ($r = 0.85$, $P < 0.001$) also exists between cadaverine and putrescine. No literature was found which describes or illustrates these statistical correlations. Ababouch et al. (1991) reported that cadaverine levels were as high as that of histamine in sardines stored at 8°C. When histamine levels are greater than DAL, fish decomposition is assumed to have occurred. Because histamine increase is generally accompanied by cadaverine and putrescine accumulation, it is reasonable to state that cadaverine and putrescine are good indicators of mahi-mahi quality deterioration. This correlation of histamine and cadaverine formation is of significance in view of the fact that histamine is known to be an unreliable indicator of fish quality, and that cadaverine is generally present in bacterially decomposed fish (Rogers and Staruszkiewicz, 1997) and meats (Slemr and Beyermann, 1984). In addition, cadaverine undergoes considerable changes in its levels during storage, which makes it a reliable indicator. It is reasonable to suggest also that cadaverine be investigated as a general biochemical indicator of other seafoods together with TVB-N, which itself is used for a wide number of fish species.

Histamine with APC and histamine producers. Figure 4-20 shows that there is a weak correlation ($r = 0.54$) between histamine formation and the growth of the APC population. A much stronger correlation ($r = 0.76$) exists between histamine producers and histamine levels. This is evidence that a high microbial population in fish is no indication of the presence of histamine levels greater than DAL, and that the presence of histamine producing bacteria is also important for histamine formation in fish (Taylor, 1986), but is not the only determining factor. As explained above, histamine formation is

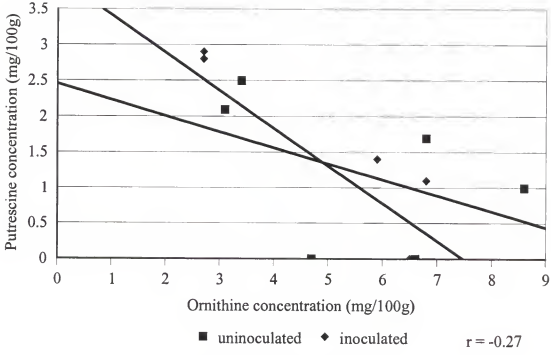


Figure 4-18. Putrescine correlation with ornithine.

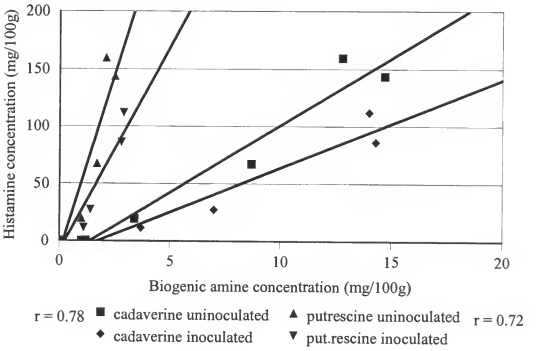


Figure 4-19. Histamine correlation with cadaverine and putrescine.

also dependent on the presence of a high enough level of histidine (Eitenmiller and De Souza, 1984).

Histamine with TVB-N. The relationship between histamine and TVB-N (Figure 4-21) is interesting simply because both histamine and TVB-N are used as fish quality indicators; both TVB-N and histamine develop later than cadaverine; both develop quickly after two days of storage; and both, at certain thresholds, are indicative of fish spoilage. There is good correlation between TVB-N and histamine ($r = 0.74$, $P < 0.001$). There is no dependence of histamine formation on TVB-N levels, and the fact that histamine and TVB-N correlation is high, is evidence that TVB-N is a good indicator of mahi-mahi spoilage, and possibly can be an indicator of the likelihood of histamine presence if the fish is a red muscle species. Kimata et al. (1953) stated that in the white muscle fish, if the only toxic substance produced in the early stage of spoilage is histamine, then freshness as determined by NH_3, a major component of TVB-N, appears to be reliable from a sanitary point of view, since large amount of NH_3 is produced before histamine is produced.

TVB-N with cadaverine and with putrescine. The correlations between TVB-N and cadaverine ($r = 0.74$, $P < 0.001$) and TVB-N with putrescine ($r = 0.80$, $P < 0.001$) are shown in Figure 4-22. This result agrees with previous investigation in this lab that showed a linear correlation between TVB-N and cadaverine and with putrescine. This relationship between TVB-N and cadaverine and between TVB-N and putrescine is evidence that TVB-N, cadaverine, and putrescine are good biochemical indicators of mahi-mahi quality. An even stronger correlation ($r = 0.85$, $P < 0.001$) was observed

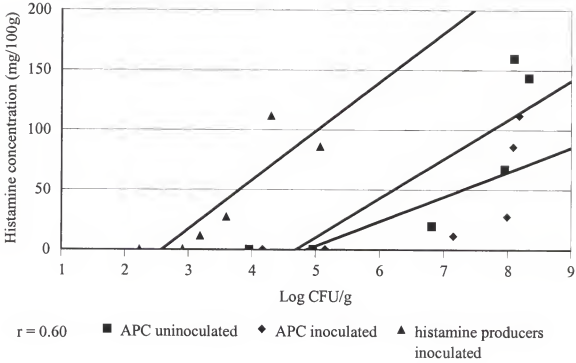


Figure 4-20. Histamine correlation with histamine producers and APC.

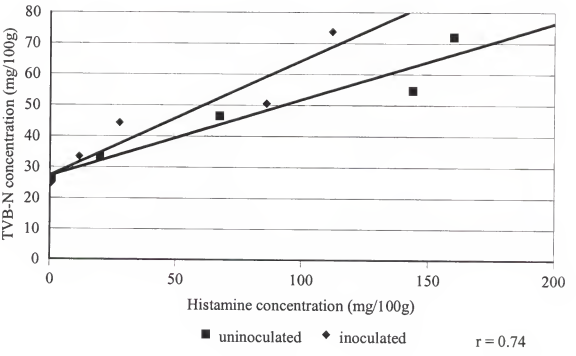


Figure 4-21. Histamine correlation with TVB-N.

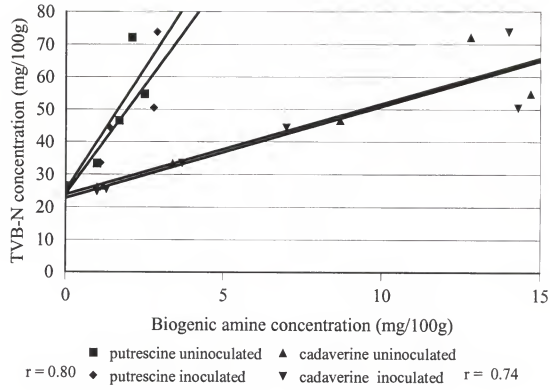


Figure 4-22. TVB-N correlation with cadaverine and putrescine.

between cadaverine and putrescine. While cadaverine formation begins from the early stages of storage, putrescine and TVB-N begin increasing at later stages. Cadaverine levels, therefore, would be indicative of both incipient and late spoilage, and putrescine and TVB-N of advanced spoilage.

Yamanaka (1990) stated that cadaverine formation agrees well with the degree of decomposition in several particular species. He also recommended cadaverine as a useful index for fish spoilage. Sims et al. (1992) also reported good correlation between expert sensory evaluations and levels of cadaverine and putrescine. They concluded that

whenever putrescine and cadaverine were greater than background levels in canned tuna, it was evident that the material had reached advanced decomposition prior to heating.

TVB-N with sensory odor. Figure 4-23 shows the correlation ($r = -0.84$, $P < 0.001$) of TVB-N with sensory odor of mahi-mahi. This further illustrates the fact that TVB-N, already known to be a good quality indicator of several fish species (Tennyson, 1999), is a good indicator of quality for chilled mahi-mahi. Connell and Shewan (1980) sensory changes during spoilage and deterioration. Two of its main disadvantages, however, are the need to standardize the conditions of volatization, and its insensitivity to early quality deterioration (Woyewoda et al., 1986; Connell and Shewan, 1980).

TVB-N with lysine. An interesting correlation ($r = -0.69$, $P < 0.001$) was observed between TVB-N and lysine (Figure 4-24). As TVB-N levels increased, the levels of lysine decreased proportionally. This probably indicates the role of lysine in the development of TVB-N, but results also show that there is good correlation ($r = -0.74$, $P < 0.001$) between APC and lysine as there is between TVB-N and microbial levels. It should be pointed out, however, that TVB-N is comprised also of TMA, and DMA produced from TMAO breakdown (Connell and Shewan, 1980). The correlation may also indicate that lysine can be a potential biochemical indicator of mahi-mahi quality. What is known, however, is that ammonia is a major component of TVB-N (Wekell et al., 1987), and is also generated from glutamine, ornithine and lysine (Barker, 1981) produced as a result of amino acid deamination. Lysine contains an ϵ-NH_2 group which, even if this amino acid is in peptide linkage, can be deaminated. Thus, bound lysine may be a source of NH_3 groups present in TVB-N. Only a few researchers have reported on

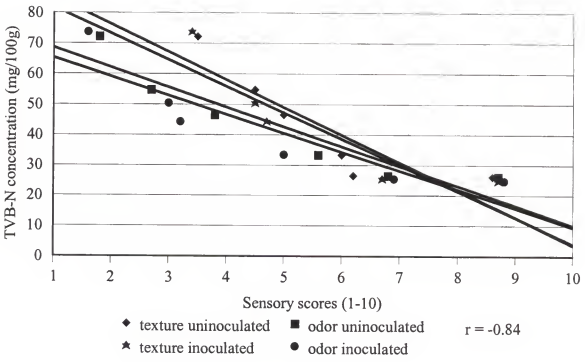


Figure 4-23. TVB-N correlation with sensory odor and texture.

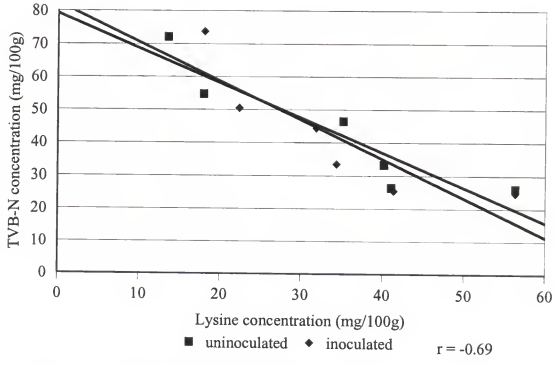


Figure 4-24. TVB-N correlation with lysine.

the changes in lysine levels during the storage of fish (Ababouch et al., 1991; Shewan and Jones, 1957; Ranke, 1957). Because it was observed that lysine correlates well with TVB-N and cadaverine, it may be worthwhile to investigate lysine as a potential quality indicator for refrigerated mahi-mahi, and possibly other species. Besides, lysine is well known as one of the essential amino acids which, if significant losses occur during storage, can reduce the protein quality of fish.

It has also been observed that the loss of lysine correlates ($r = -0.63$, $P < 0.001$) with histamine (Figure 4-25A) and with APC ($r = -0.74$) (Figure 4-25B). For what these may be worth, they are simply interesting observations, and may simply indicate that lysine plays an important role during microbial spoilage and histamine development (Morris and Fillingame, 1974).

APC with sensory odor. Figure 4-26 shows that APC population correlates ($r = -0.923$) with sensory odor. This is similar to Hanna's report (1992) that the microbial number in fish correlates with sensory scores, though he did not give a correlation value. The fact that TVB-N correlates well with sensory odor is interesting, because APC is also shown to correlate well with sensory odor (see Figure 4-26). This probably reflects an interdependent biochemical relationship that exists between APC growth, TVB-N formation, and sensory odor.

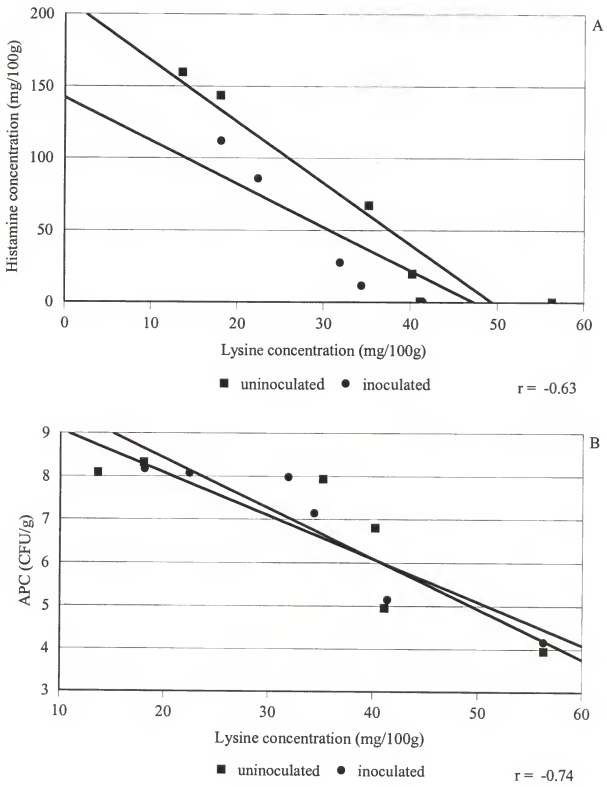


Figure 4-25. Lysine correlation with (A) histamine and (B) APC.

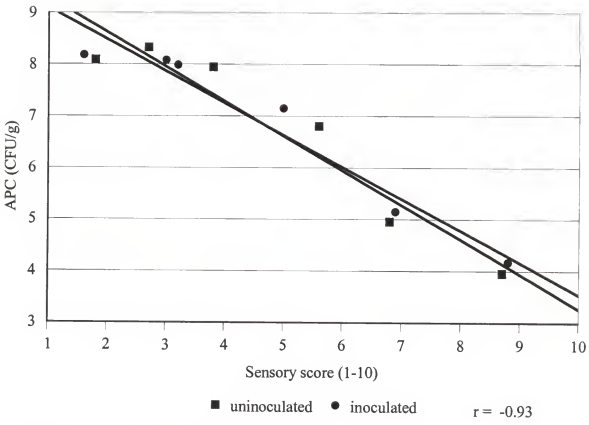


Figure 4-26. APC correlation with sensory odor.

Hypothesis. In conclusion, the results illustrate that significant changes occur in the levels of histidine, lysine, ornithine and glutamine, during the chilled storage of mahi-mahi. During prolonged (greater than 3 days) storage of mahi-mahi at 7°C, significant levels of histamine, cadaverine and TVB-N are formed. Concurrent with histamine and cadaverine formation, is the significant increase in putrescine, though not in high levels. Because of the good correlation between histamine, cadaverine, putrescine and TVB-N, it can be concluded that these are good biochemical indicators of quality deterioration in

chilled mahi-mahi, and that the levels of cadaverine, putrescine, and TVB-N, can potentially signal the likely presence of histamine in mahi-mahi, because of their high correlations. Cadaverine by itself is a good indicator of both incipient and late spoilage, and TVB-N and putrescine good indicators of advanced spoilage.

CHAPTER 5
SUMMARY AND CONCLUSIONS

Two groups of mahi-mahi (*Coryphaena hippurus*) were stored at 7°C for 10 days. One group (B) was dipped for two minutes in an inoculum of *Morganella morganii*. Group B was inoculated to ensure that histamine formation occurred in fish so that the objectives of the study could be answered, and to serve as a model to see if there would be any difference in the development of biogenic amines from uninoculated fish. Samples were taken on days 0, 2, 4, 6, 8, and 10 for analysis.

The specific objectives of the study were (i) to quantify the levels of some biogenic amines formed in mahi-mahi during storage at 7°C; (ii) to determine how the levels of the biogenic amines correlate with their precursor free amino acids, TVB-N, and sensory evaluation; (iii) in oder to acheive our objectives it was necessary to to develop a HPLC method which would enable quick analysis of free amino acids in fish. In addition, AromaScan analysis was done.

The results showed that the levels of histamine formed were significantly ($P < 0.05$) high relative to the levels of cadaverine and putrescine. Cadaverine levels were significantly higher ($P < 0.05$) than putrescine. Cadaverine formation began earlier than putrescine and histamine and its levels increased steadily. Putrescine and histamine levels began to increase after a few days in storage. The increase in the levels of these biogenic

amines were accompanied by a general decrease in the levels of their precursor amino acids.

Histamine levels varied widely among the fish in the same group, and its levels were significantly lower ($P < 0.05$) in the inoculated group of fish, even though there was no significant difference in the levels of its precursor in the said groups. There was poor correlation ($r = -0.43$) between the levels of histamine formed and the decrease in levels of histidine, even though histamine was formed directly as a result of histidine decarboxylation. Good correlation existed between histamine development and cadaverine ($r = 0.78$, $P < 0.0010$), histamine and putrescine ($r = 0.72$, $P < 0.001$), and between histamine and TVB-N ($r = 0.73$, $P < 0.001$).

Cadaverine levels between the fish varied less than did histamine, and there was no significant difference ($P < 0.05$) in the levels between the two groups of fish. This is probably because of the wide range of bacteria capable of producing cadaverine. Cadaverine increase began early and proved to be a good indicator of both incipient and late spoilage. The increase in cadaverine levels correlated ($r = -0.62$, $P < 0.001$) with the decrease in levels of lysine, its precursor amino acid. Good correlation ($r = 0.74$, $P < 0.001$) also existed between cadaverine and TVB-N, between cadaverine and putrescine ($r = 0.85$, $P < 0.001$), and between cadaverine and sensory odor. Cadaverine, therefore, can serve as a good biochemical indicator of quality in chilled mahi-mahi. In addition, because cadaverine formation readily occurs in protein foods, it has the potential to be a good biochemical quality indicator for a wide range of fish, i.e. both red and white muscle

fish and shellfish. If adopted by industry, standards will be needed and new and rapid tests for cadaverine will have to be developed.

Putrescine levels were low and very limited changes occurred in its levels during the storage period. There was no significant difference ($P < 0.05$) between the levels in the inoculated and uninoculated groups of fish. This is probably because of the wide range of bacteria capable of producing putrescine. Poor correlation ($r = -0.27$) existed between putrescine and its precursor, ornithine. Good correlation existed, however, between putrescine and TVB-N ($r = 0.80$, $P < 0.001$). By itself, putrescine may not be a good indicator of quality in some fish species because of limited changes in its levels. Other authors have shown, however, that it is a useful component in a summation index of biogenic amines (Mietz and Karmaz, 1978; Slemr and Beyermann, 1984).

In this study a method was successfully developed which allows the quick analysis of free amino acids in fish. The method uses OPA precolumn derivatization and gradient elution for quantification of the amino acids. The effectiveness of the method to analyze free amino acids was demonstrated using white and red muscle tissues (Antoine et al., 1999; Antoine et al., 2000, in review), and the mahi-mahi samples used in this study.

No significant difference ($P < 0.05$) existed between the levels of histidine in the uninoculated and inoculated groups of fish. This was because each of the two groups got one of the fillets of each fish. Free histidine levels varied considerably, however, among mahi-mahi even though they were caught on the same day, in the same region. The initial histidine levels were high, but during the period of storage the levels decreased

significantly ($P < 0.05$), and the loss in histidine was not accounted for solely by the histamine formed, because other metabolic derivatives are formed from histidine .

The levels of lysine were higher than those of ornithine and glutamine, but lower than those of histidine. There was no significant difference ($P < 0.05$) between the levels of lysine in the uninoculated and the inoculated fish samples. The decrease in lysine levels during the period of storage was significant ($P < 0.05$), and linear correlation of $r = -0.69$ ($P < 0.001$) was observed between lysine and TVB-N, and between lysine and histamine ($r = -0.63$, $P < 0.001$) development. In addition to lysine being an essential amino acid, it may be possible that it can be used as an indicator of freshness and spoilage quality for chilled mahi-mahi, and possible other fish species.

Ornithine and glutamine levels in mahi-mahi were low, relative to lysine levels. There was no significant difference ($P < 0.05$) in their levels between the uninoculated and the inoculated mahi-mahi. The changes in the levels of these amino acids were limited, and poor correlation was observed between ornithine and putrescine ($r = -0.27$).

Cadaverine and putrescine tend to always be present in spoiled fish, whereas histamine is not (Rogers and Staruszkiewicz, 1997; Rodriguez-Jerez et al., 1994; Middlebrooks et al., 1988). The results, therefore, would support the recommendation to the AOAC International for adoption of the GC method for cadaverine and putrescine analysis (Rogers and Staruszkiewicz, 1997).

Good correlation ($r = -0.84$) existed between sensory odor and TVB-N, and between sensory and APC ($r = -0.92$). These correlations illustrate why the FDA use of sensory odor of volatiles from suspected decomposed tuna for over two decades were

useful. The correlations also show that use of TVB-N, in conjunction with sensory odor, is a good objective fish quality test.

Overall, the results provide evidence that cadaverine, TVB-N, and putrescine are good indicators of mahi-mahi quality. TVB-N and putrescine are good indicators of late spoilage, and cadaverine is a good indicator of both incipient and late spoilage in mahi-mahi.

Recommendations. The following are some recommendations arising from the results of this study.

Cadaverine and TVB-N together can be used as objective chemical indicators of mahi-mahi freshness quality and spoilage.

Rapid microscale test methods should be developed for analysis of cadaverine, lysine, and TVB-N.

Other biogenic amines should be investigated as possible species-specific biochemical indicators, for example, agmatine for shrimp, and it is suggested that such investigation should probably consider the molar ratios of the amino acids and their derived biogenic amines.

Any considerable loss of lysine could be indicative of bacterial enzymatic activity because lysine is readily utilized by a wide range of bacteria. In addition, loss of lysine during refrigerated storage correlates well with TVB-N, histamine and cadaverine formation. Because any significant loss in lysine represents a reduction in protein quality of fish, and because it is known that considerable loss of lysine occurs during autolytic

changes in fish (Shewan and Jones, 1957), it would be useful to further investigate lysine as a useful indicator of fish freshness quality deterioration.

The experiment answered the questions posed in its objectives and raised some new insights for the field of seafood chemistry. Much work will be needed to establish threshold levels for cadaverine, putrescine, TVB-N, and possibly lysine, and in the various fish species for which they would be good indicators of freshness and spoilage.

APPENDIX A

NUMBER OF REPORTED SCOMBROTOXIN OUTBREAKS, CASES AND DEATHS IN THE USA, INCLUDING GUAM, PUERTO RICO AND US VIRGIN ISLANDS

YEAR	OUTBREAKS		CASES		DEATHS	
	No.	%	No.	%	No.	%
88	16	3.5	65	0.4	1	5.3
89	17	3.4	80	0.5	0	0
90	11	2.1	194	1.0	0	0
91	17	3.2	40	0.3	0	0
92	15	3.7	135	1.2	0	0

N.B.: % indicates total number of reported foodborne disease outbreaks, cases and deaths.

Most outbreaks occur as a result of eating at delicatessen, cafeteria or restaurants.

1988 - 8 outbreaks due to improper temperature
2 due to unsafe sources

1989 - 7 outbreaks - improper temperature
1 inadequate cooking
1 unsafe source

1990 - 7 outbreaks due to improper temperature
1 due to contaminated equipment
1 due to unsafe source

1991 - 13 outbreaks due to improper temperature
1 due to contaminated equipment
5 due to unsafe sources

1992 - 1 due to unsafe source

Source: CDC Morbidity and Mortality Weekly Report, 1996.

APPENDIX B
SCOMBROID FISH POISONING IN THE UNITED STATES
REPORTED TO CDC, 1978-1987

State	Outbreaks	Cases
Alaska	3	17
Arizona	3	7
California	18 (12%)	69 (9%)
Connecticut	8	47
District of Columbia	1	3
Florida	1	20
Hawaii	45 (29%)	170 (23%)
Idaho	1	4
Illinois	3	35
Indiana	1	4
Kentucky	1	7
Maine	3	54
Maryland	1	10
Michigan	3	25
Minnesota	1	24
Nebraska	1	10
New Jersey	4	42
New Mexico	1	2
New York	30 (19%)	122 (16%)
North Carolina	1	10
Pennsylvania	2	4
Texas	2	11
Vermont	3	6
Virginia	2	13
Virgin Islands	1	5
Washington	16	35
Wisconsin	1	1
Total:	157	757

Source: *Naturally Occurring Fish and Shellfish Poisons*, In Seafood Safety, 1991.

APPENDIX C
SIGNIFICANT HAZARDS ASSOCIATED WITH SEAFOOD[a]

	Reported	Upperbound	Estimated
Anasakis	1	100	100
Campylobacter jejuni	2	200	200
Ciguatera	800	8,000	800
Clostridium botulinum	4	4	4
Clostridium perfringens	7	70	70
Diphyllobothrium latum	([1])	1,000	1,000
Giardia	3	50	30
Hepatitis A virus	9.2	6,700	92
Neurotoxic shellfish poisoning	48	48	48
Norwalk virus	12.4	30,000	12,400
Other Vibrios	43	10,000	10,000
Paralytic shellfish poisoning	13.4	13.4	13.4
Salmonella nontyphi	2	2,750	200
Scombrotoxin	796	21,500	7,960
Shigella	7	100	70
Vibrio vulnificus	24	48	48
Total	1,772	80,389	33,035

[1] Unknown
[a]All Seafood Sources Combined - Recreational and Commercial.

Source: FDA, 1994, Vol. 59, No. 19, 4190.

APPENDIX D

SENSORY DESCRIPTORS FOR JUDGING THE QUALITY ATTRIBUTES OF RAW (FRESH AND STORED) MAHI-MAHI FILLET

Attributes:	*Defect degree & score*	*Descriptors*
Appearance:		
Color	Very bright 10-9	Bright attractive pink/red, clear translucent juice, excellent, rich and attractive appearance, preferred product.
	Bright 8-7	Fair change in initial color, possible drying at edges, thickening of juice.
	Dull 6-5	Considerable or obvious color change, slimy or tacky surface, fair appearance, less appealing.
	Brown 4-1	Reject due to obvious slime, dull or beige color, loss of appeal.
Gaping	None 10-9	Intact, no separation of tissue, connective tissue not seen.
	Slight 8-7	Small amount of tissue separation, some visible connective tissue.
	Moderate 6-5	Obvious breaking apart of tissue, wide separations or openings in tissue, clearly visible connective tissue.
	Excessive 4-1	Reject due to excessive breaking or falling apart of tissue.
Texture:	Subjective measure of meat firmness and/or resilience, determined by firmly pressing fish with index finger and observing the extent of deformation of yielding.	
	Very firm 10-9	Immediately returns to original shape. Resilient. Firm.
	Firm 8-7	Slow, delayed return to original shape.
	Soft 6-5	Some deformation, soft tissue.
	Very soft 4-1	Very soft tissue, distinct deformation, fragmentation. Breaks upon pressing, reject.

Appendix D cont. Sensory descriptors for judging the quality attributes of raw (fresh and stored) mahi-mahi fillet.

Odor:

Very fresh 10-9	Very mild fresh aroma, seaweedy aroma.
Slightly fresh 8-7	Evident fishy odor, typical.
Moderately fishy 6-5	Objectionable fish odor, evidence of spoilage byproducts.
Ammoniacal 4-1	Distinct spoilage odor, putrid, ammonia-like, reject.

APPENDIX E
SENSORY EVALUATION BALLOT FOR FISH FRESHNESS QUALITY ASSESSMENT

Product: mahi-mahi Name: Date: 2-12-99

Assess the contents of each of the pouches, and score each attribute using a scale of 1 to 10, where 1 indicates low rating and 10 a high rating. Circle the score given to each sample on the corresponding line.

Sample Code: _____

Appearance

color	*brown*				*dull*		*bright*		*very bright*	
	1	2	3	4	5	6	7	8	9	10
gaping	*excessive*				*moderate*		*slight*		*none*	
	1	2	3	4	5	6	7	8	9	10
Texture	*very soft*				*soft*		*firm*		*very firm*	
	1	2	3	4	5	6	7	8	9	10
Odor	*ammoniacal*				*moderately fishy*		*slightly fishy*		*very fresh*	
	1	2	3	4	5	6	7	8	9	10

Sample Code: _____

Appearance

color	*brown*				*dull*		*bright*		*very bright*	
	1	2	3	4	5	6	7	8	9	10
gaping	*excessive*				*moderate*		*slight*		*none*	
	1	2	3	4	5	6	7	8	9	10
Texture	*very soft*				*soft*		*firm*		*very firm*	
	1	2	3	4	5	6	7	8	9	10
Odor	*ammoniacal*				*moderately fishy*		*slightly fishy*		*very fresh*	
	1	2	3	4	5	6	7	8	9	10

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BIOGRAPHICAL SKETCH

Francis was born in Grenada, the Spice Island of the Caribbean. After graduating from the Grenada Boys Secondary School he served four years in the military. Francis then obtained a scholarship to study in Russia, where he received a master's degree in food technology at the Krasnodar Polytechnical Institute. Upon return to Grenada, he taught for two years at the Grenada National College. During that time, he consulted at the Grenfruit Women's Cooperative and Barte Meats Processing Plant. He then went on to do a post graduate diploma in post harvest technology, specializing in seafoods, at Humberside Polytechnical College (University of Lincolnshire & Humberside), Grimsby, England. In 1992 Francis entered the University of Florida to pursue a Doctor of Philosophy degree in food science. During his years at UF, he taught microbiology for seven semesters at Santa Fe Community College and tutored several students in mathematics and biochemistry. Francis is a member of the Gamma Sigma Delta Honorary Society, IFT and ACS. He has traveled throughout Europe, the Caribbean and the US and has learned to adapt to various cultures. Francis will graduate in August 2000. He is married to Joline, whom he first met in Grenada, where they worked together on a solar drying project.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Maurice R. Marshall, Jr., Chair
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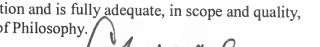
Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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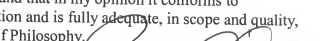
Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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